(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 February 2004 (19.02.2004)

PCT

(10) International Publication Number
WO 2004/015113 A2

(51) International Patent Classification[7]: C12N 15/62

(21) International Application Number: PCT/EP2003/008710

(22) International Filing Date: 6 August 2003 (06.08.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/401,833 7 August 2002 (07.08.2002) US

(71) Applicant: **AVENTIS BEHRING GMBH** [DE/DE]; Intellectual Property/Legal, Postfach 1230, 35002 Marburg (DE).

(72) Inventors: **JÜRS, Mathias**; Im Anger 31 b, 65529 Waldems Reichenbach (DE). **WEIMER, Thomas**; Richard-Wagner-Strasse 8, 35075 Gladenbach (DE). **HAUSER, Hans-Peter**; Feuerdornweg 6, 35041 Marburg (DE). **SLEEP, Darrell**; 66 Ladybay Road, West Bridgford, Nottingham NG2 5DS (GB).

(81) Designated States *(national)*: AU, CA, JP, KR.

(84) Designated States *(regional)*: European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AU, CA, JP, KR, European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR)*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for the following designations AU, CA, JP, KR, European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR)*

— *of inventorship (Rule 4.17(iv)) for US only*

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ALBUMIN-FUSED CILIARY NEUROTROPHIC FACTOR

(57) Abstract: The invention relates to a fusion protein comprising an albumin, or a fragment or a variant or a derivative thereof and at least one biologically active peptide or protein which activates the ciliary neurotrophic factor (CNTF) receptor, or a fragment or variant or a derivative thereof.

WO 2004/015113 A2

Albumin- Fused Ciliary Neurotrophic Factor

Field of the Invention

The invention relates to a fusion protein comprising an albumin, or a fragment or a variant or a derivative thereof and at least one biologically active peptide or protein which activates the ciliary neurotrophic factor (CNTF) receptor, or a fragment or variant or a derivative thereof.

Background of the Invention

Regulation of daily energy homeostasis stands mainly under the central control of a few discrete nuclei [1] in the basal hypothalamus (ventromedial nucleus, dorsomedial nucleus, paraventricular nucleus, and lateral hypothalamus), but there are also other central nervous structures (cerebral cortex, limbic region, brainstem, pituitary gland, autonomic preganglionic neurons, dorsal vagal complex) as well as peripheral nervous structures (sympathetic preganglionic neurons) involved [2].

Beside the central and peripheral nervous regulation, peripheral organs involved in the balance of energy homeostasis are the gastrointestinal tract (stomach, gut), the pancreas, the adipose tissue, the muscle tissue, the adrenal glands and the thyroid gland.

The process of regulation is complex and peripheral organs such as the gastrointestinal tract can release hormones after food intake (e.g. CCK (cholecystokinin)), which cause a decrease of appetite-increasing hormones in the hypothalamus. Furthermore, leptin, released by fat tissue after food intake, has a negative regulatory effect on e.g. NPY (Neuropeptide Y) which is one of the major centrally active appetite-inducing hormones. Centrally released hormones, on the other side, may have a peripheral effect as well (e.g. β_3-adrenergic agonists,

uncoupling protein (UCPs)) increasing thermogenesis. The interested reader is referred to actual reviews covering the whole spectrum [1-6].

AXOKINE® (REGENERON, Terrytown, NY, USA) is a mutant version of the CNTF. AXOKINE® is the truncated form of CNTF where the last 15 C-terminal amino acids have been removed. To enhance the stability of the molecule, glutamine is replaced by arginine at position 63 and the free cysteine at position 17 is replaced by alanine [7].

By chance the weight-reducing effect of CNTF was discovered during clinical trials in subjects suffering from motor-neuron disease [8]. Further studies revealed that the mechanism of action provided by CNTF to induce loss of weight is similar to leptin with the difference that CNTF is also active in diet-induced obesity [7]. Studies in animals using AXOKINE® confirmed the weight-loss inducing capacity by this CNTF-mutant similar to the CNTF-mechanism.

CNTF has a negative regulatory effect on the synthesis of NPY, Agouti-related peptide (AGRP) and gamma-aminobutyric acid (GABA), all known to stimulate feeding.

CNTF was shown to cross the blood brain barrier (BBB) in an intact form [10]. Recently it was shown that CNTF is transported via a saturable transport system with a rate of entry K_i of 4.60 ($\pm$0.78) x 10^{-4} mL/g min [11].

The BBB is a highly regulated barrier to molecules from the blood preventing them to enter the brain tissue [13]. It is formed by brain capillary endothelial cells.

From Lambert et al. [7] we know that AXOKINE® worked in leptin deficient (ob/ob) and wild-type (diet-induced obesity, DIO) mice. The most effective dose was 300µg/kg b.w. of AXOKINE®, but effects were also observed with 100µg/kg b.w. Weight loss achieved was mainly due to loss of fat tissue, avoiding loss of lean body mass.

Furthermore, there was no rebound effect in mice treated with AXOKINE® whereas mice not treated with AXOKINE® and receiving the diet the AXOKINE® treated animals consumed (pair fed group), quickly regained their original weight.

Phase I data were published by Guler et al. in the International Journal of Obesity [14]. AXOKINE® was tolerated well, no subjects dropped out and the majority of all adverse events (AE) were considered to be "mild". Dose limiting toxicities were vomiting and nausea in part A at 16 µg/kg b.w. Injection site reactions were the most frequently reported AE in the drug treated subjects, followed by decreased appetite, nausea, headache, and diarrhoea. Herpetiform mouth lesions were noted in some subjects.

One subject suffered a transient Bell's palsy (palsy of the VIIth cranial nerve, the facial nerve, where the mimic muscles of the face get paralysed) 10 days after the end of treatment with AXOKINE® at 1 µg/kg b.w./day. At the higher doses, increased C-reactive protein and erythrocyte sedimentation rate (ESR), and decreased serum Fe^+ were noted. In a dose-dependent fashion, heart rate increased and body temperature tended to be higher.

A multicenter, randomised, double-blind, placebo-controlled, dose-ranging phase II study [15] involving 170 severely- or morbidly obese patients has evaluated that patients receiving the optimal dose of AXOKINE® (1.0 µg/kg) over the 12-week treatment period averaged a 10-pound greater [16] weight loss than placebo recipients ($p < 0.001$).

Weight loss was maintained for 4 months after the last administration of AXOKINE® in patients from the 8-week treatment group [17, 18]. No serious adverse events were reported. The most frequently reported adverse event was dose-dependent, mild injection site reaction (site redness) that occurred in all patients, including placebo group. The administration of AXOKINE® was associated with cough and nausea, which occurred most frequently after the 2.0µg/kg b.w. dose of the agent. No increase in herpes simplex virus infections was observed in AXOKINE® recipients compared with placebo. Comparable proportions of AXOKINE®, and (58-74%), and placebo (61%), recipients completed the full 12-week study.

In a phase III placebo-controlled study 1467 AXOKINE- treated subjects and 501 placebo-treated subjects demonstrated that:

- AXOKINE®treatment, when compared with placebo, achieved statistical significance with regard to both primary endpoints of the study:
    - A greater proportion of AXOKINE® -treated patients lost at least 5% of their initial body weight compared with placebo-treated patients (25.1% vs. 17.6%, p<.001)
    - Participants receiving AXOKINE®experienced a greater average weight loss than those receiving placebo (6.2 lbs vs. 2.6 lbs, p<.001)
- AXOKINE®treatment achieved statistically significant results in two of the three secondary endpoints, such as proportion of subjects losing at least 10% of their initial body weight (11.3% vs. 4.2%, p <.001)
- AXOKINE® treatment was generally well-tolerated. Adverse events were generally characterized as mild to moderate and no pattern of serious or severe adverse events emerged. The most notable adverse effects as compared with placebo were injection site reactions, nausea and cough, which were largely characterized as mild
- AXOKINE®-associated weight loss was limited by the development of antibodies beginning after about three months of AXOKINE® treatment. However, more than 30% of the total 1467 subjects treated with AXOKINE®did not develop antibodies by the end of one year

Summary of the Invention

In one aspect of the invention, the invention is a fusion protein comprising an albumin, in particular a human serum albumin, or a fragment or a variant or a derivative thereof, which has an albumin activity, and at least one biologically active peptide or protein which activates the ciliary neurotrophic factor (CNTF) receptor, or a fragment or variant or a derivative thereof.

In different embodiments, CNTF or albumin may be a fragment or a derivative, or both as in the case of AXOKINE®, or a variant. The albumin fusion protein may be a therapeutic agent.

In another aspect, the invention is a method for extending the half-life of CNTF in a mammal. The method entails linking a CNTF to an albumin to form an albumin-fused CNTF and administering the albumin-fused CNTF to a mammal. Typically, the half-life of the albumin-fused CNTF is extended by at least 2-fold to at least 50-fold over the half-life of the CNTF lacking the linked albumin.

By using either the transport system for CNTF or unspecific transport systems across the blood brain barrier (BBB) like e.g. transcytosis, the intracerebral concentration of albumin fused AXOKINE® is expected to be increased. Due to the increased plasma concentration of the albumin-fused AXOKINE® over time at the BBB compared to the non-fused AXOKINE® a higher influx of albumin-fused AXOKINE®will take place via transcytosis.

Further, the invention entails a method for treating obesity in a mammal. The method comprises linking CNTF to an albumin to form an albumin-fused CNTF and administering the albumin-fused CNTF to the mammal. The invention also encompasses a method for potentially minimizing side effects (e.g. nausea, headache) associated with the treatment of a mammal with CNTF in moderately higher concentrations. The method comprises linking said CNTF to an albumin to form an albumin-fused CNTF and administering said albumin-fused CNTF to said mammal.

Brief Description of the Drawings

Figure 1. Pharmacokinetics of non-fused AXOKINE® in rabbits (i.v.)

Figure 2. Pharmacokinetics of C- and N-terminal fused AXOKINE® in rabbits (i.v.)

Figure 3. Pharmacokinetics of C- and N-terminal fused AXOKINE® in rabbits (s.c.)

Figure 4. Weight loss curve of leptin deficient mice treated with non-fused AXOKINE®

Figure 5. Weight loss curve of leptin deficient mice treated with C-terminal fused AXOKINE®

Figure 6. Weight loss curve of wild type mice treated with non-fused AXOKINE®

Figure 7. Weight loss curve of wild type mice treated with C-terminal fused AXOKINE®

Figure 8. Amino acid sequence of the mature C-terminal AXOKINE® (Seq. ID: 1)

Figure 9. Amino acid sequence of the mature C-terminal rHA-3xFLAG- (cleavable) AXOKINE® (Seq. ID: 2)

Figure 10. Amino acid sequence of the mature N-terminal AXOKINE® (Seq. ID: 3)

Figure 11. Map of the C-terminal fused AXOKINE®

Figure 12. Map of the C-terminal rHA-3xFLAG- (cleavable) AXOKINE®

Figure 13. Map of the N-terminal fused AXOKINE®

Figure 14. Weight loss curve of leptin deficient mice treated every third day with non-fused AXOKINE® and C-terminal fused AXOKINE®

Figure 15. Weight loss curve of leptin deficient mice treated daily with non-fused AXOKINE® and C-terminal fused AXOKINE®

Detailed Description of the Invention

Definitions:

Ciliary neurotrophic factor (CNTF) means any molecule which is an analogue, homologue, fragment, or a derivative of naturally occurring CNTF which possesses a single biological activity of the naturally occurring CNTF. A preferred CNTF is AXOKINE®. Another CNTF mutant (Ser166Asp/Gln167His) has been described in the European Application WO 98/22128, which, from position 159 to position 178, has the following amino acid sequence:

Leu Lys Val Leu Gln Glu Leu Asp His Trp Thr Val Arg Ser Ile His Asp Leu Arg Phe (159-178; Seq. ID: 4)

AXOKINE® is a mutant version of the CNTF. AXOKINE® is the truncated form of CNTF where the last 15 c-terminal amino acids have been removed. To enhance the stability of the molecule, glutamine is replaced by arginine at position 63 and the free cysteine at position 17 is replaced by alanine [7]

N-terminal-AXOKINE® is a fusion of the C-terminal end of AXOKINE® to the N-terminal end of human serum albumin as described in example 1.

C-terminal-AXOKINE® is a fusion of the N-terminal end of AXOKINE® to the C-terminal end of human serum albumin as described in example 1.

Cleavable AXOKINE® as described in example 1 is a C-terminal fusion of AXOKINE® to human serum albumin which has an enterokinase cleavage site between the CNTF portion and albumin which was used to generate cleaved or **non-fused AXOKINE®** which was used as a control to the N- and C-terminal fusions.

Albumin

The terms, human serum albumin (HSA) and human albumin (HA) are used interchangeably herein. The terms, "albumin and "serum albumin" are broader, and encompass human serum albumin (and fragments and variants thereof) as well as albumin from other species (and fragments and variants thereof).

As used herein, "albumin" refers collectively to albumin protein or amino acid sequence, or an albumin fragment or variant, having one or more functional activities (e.g., biological activities) of albumin. In particular, "albumin" refers to human albumin or fragments thereof (see EP 201 239, EP 322 094 WO 97/24445, WO95/23857) especially the mature form of human albumin as shown in Figure 15 (SEQ ID NO:18) of WO 01/79480, hereby is incorporated in its entirety by reference herein, or albumin

from other vertebrates or fragments thereof, or analogs or variants of these molecules or fragments thereof.

This sequence of Fig. 15 of WO 01/79480 is in this application referred to as the "WO '480 sequence".

The human serum albumin protein used in the albumin fusion proteins of the invention contains one or both of the following sets of point mutations with reference to WO '480 SEQUENCE: Leu-407 to Ala, Leu-408 to Val, Val-409 to Ala, and Arg-410 to Ala; or Arg-410 to Ala, Lys-413 to Gln, and Lys-414 to Gln (see, e.g., International Publication No. WO95/23857, hereby incorporated in its entirety by reference herein). In other embodiments, albumin fusion proteins of the invention that contain one or both of above-described sets of point mutations have improved stability/resistance to yeast Yap3p proteolytic cleavage, allowing increased production of recombinant albumin fusion proteins expressed in yeast host cells.

As used herein, a portion of albumin sufficient to prolong or extend the *in vivo* half-life, therapeutic activity, or shelf-life of the CNTF refers to a portion of albumin sufficient in length or structure to stabilize, prolong or extend the *in vivo* half-life, therapeutic activity or shelf-life of the CNTF portion of the albumin fusion protein compared to the *in vivo* half-life, therapeutic activity, or shelf-life of the CNTF in the non-fusion state. The albumin portion of the albumin fusion proteins may comprise the full length of the HA sequence as described above, or may include one or more fragments thereof that are capable of stabilizing or prolonging the therapeutic activity. Such fragments may be of 10 or more amino acids in length or may include about 15, 20, 25, 30, 50, or more contiguous amino acids from the HA sequence or may include part or all of specific domains of HA.

The albumin portion of the albumin fusion proteins of the invention may be a variant of normal HA. The CNTF portion of the albumin fusion proteins of the invention may also be variants of the Therapeutic proteins as described herein. The term “variants” includes insertions, deletions and substitutions, either conservative or non conservative, where such changes do not substantially alter one or more of the oncotic, useful ligand-binding and non-immunogenic properties of albumin, or the

active site, or active domain which confers the therapeutic activities of the Therapeutic proteins.

In particular, the albumin fusion proteins of the invention may include naturally occurring polymorphic variants of human albumin and fragments of human albumin, for example those fragments disclosed in EP 322 094 (namely HA (Pn), where n is 369 to 419). The albumin may be derived from any vertebrate, especially any mammal, for example human, cow, sheep, or pig. Non-mammalian albumins include, but are not limited to, hen and salmon. The albumin portion of the albumin fusion protein may be from a different animal than the CNTF portion.

Generally speaking, an HA fragment or variant will be at least 100 amino acids long, optionally at least 150 amino acids long. The HA variant may consist of or alternatively comprise at least one whole domain of HA, for example domains 1 (amino acids 1-194 of WO '480 SEQUENCE), 2 (amino acids 195-387 of WO '480 SEQUENCE), 3 (amino acids 388-585 of WO '480 SEQUENCE), 1 + 2 (1-387 of WO '480 SEQUENCE), 2 + 3 (195-585 of WO '480 SEQUENCE) or 1 + 3 (amino acids 1-194 of WO '480 SEQUENCE + amino acids 388-585 of WO '480 SEQUENCE). Each domain is itself made up of two homologous subdomains namely 1-105, 120-194, 195-291, 316-387, 388-491 and 512-585, with flexible inter-subdomain linker regions comprising residues Lys106 to Glu119, Glu292 to Va1315 and Glu492 to Ala511.

The albumin portion of an albumin fusion protein of the invention may comprise at least one subdomain or domain of HA or conservative modifications thereof. If the fusion is based on subdomains, some or all of the adjacent linker is may optionally be used to link to the CNTF moiety.

Albumin Fusion Proteins

The present invention relates generally to albumin fusion proteins and methods of treating, preventing, or ameliorating diseases or disorders. As used herein, "albumin fusion protein" refers to a protein formed by the fusion of at least one

molecule of albumin (or a fragment or variant thereof) to at least one molecule of a CNTF (or fragment or variant thereof). An albumin fusion protein of the invention comprises at least a fragment or variant of a CNTF and at least a fragment or variant of human serum albumin, which are associated with one another, such as by genetic fusion (i.e., the albumin fusion protein is generated by translation of a nucleic acid in which a polynucleotide encoding all or a portion of a CNTF is joined in-frame with a polynucleotide encoding all or a portion of albumin) to one another. The CNTF and albumin protein, once part of the albumin fusion protein, may be referred to as a "portion", "region" or "moiety" of the albumin fusion protein.

In one embodiment, the invention provides an albumin fusion protein comprising, or alternatively consisting of, a CNTF and a serum albumin protein. In other embodiments, the invention provides an albumin fusion protein comprising, or alternatively consisting of, a biologically active and/or therapeutically active fragment of a CNTF and a serum albumin protein. In other embodiments, the invention provides an albumin fusion protein comprising, or alternatively consisting of, a biologically active and/or therapeutically active variant of a CNTF and a serum albumin protein. In further embodiments, the serum albumin protein component of the albumin fusion protein is the mature portion of serum albumin.

In further embodiments, the invention provides an albumin fusion protein comprising, or alternatively consisting of CNTF and a biologically active and/or therapeutically active fragment of serum albumin. In further embodiments, the invention provides an albumin fusion protein comprising, or alternatively consisting of, a CNTF and a biologically active and/or therapeutically active variant of serum albumin. In some embodiments, the CNTF portion of the albumin fusion protein is the mature portion of the Therapeutic protein.

In further embodiments, the invention provides an albumin fusion protein comprising, or alternatively consisting of, a biologically active and/or therapeutically active fragment or variant of a CNTF and a biologically active and/or therapeutically active fragment or variant of serum albumin. In some embodiments, the invention provides an albumin fusion protein comprising, or alternatively consisting of, the mature portion of a CNTF and the mature portion of serum albumin.

In one embodiment, the albumin fusion protein comprises HA as the N-terminal portion, and a CNTF as the C-terminal portion. Alternatively, an albumin fusion protein comprising HA as the C-terminal portion, and a CNTF as the N-terminal portion may also be used.

In other embodiments, the albumin fusion protein has a CNTF fused to both the N-terminus and the C-terminus of albumin. In one embodiment, the CNTF proteins fused at the N- and C- termini are the same CNTF proteins. In another embodiment, the CNTF proteins fused at the N- and C- termini are different CNTF proteins. In another embodiment, the CNTF proteins fused at the N- and C- termini are different CNTF proteins which may be used to treat or prevent the same disease, disorder, or condition. In another embodiment, the CNTF proteins fused at the N- and C- termini are different CNTF proteins which may be used to treat or prevent diseases or disorders which are known in the art to commonly occur in patients simultaneously.

In addition to albumin fusion protein in which the albumin portion is fused N-terminal and/or C-terminal of the CNTF portion, albumin fusion proteins of the invention may also be produced by inserting the CNTF or peptide of interest into an internal region of HA. For instance, within the protein sequence of the HA molecule a number of loops or turns exist between the end and beginning of α-helices, which are stabilized by disulphide bonds. The loops, as determined from the crystal structure of HA (PDB identifiers 1AO6, 1BJ5, 1BKE, 1BM0, 1E7E to 1E7I and 1UOR) for the most part extend away from the body of the molecule. These loops are useful for the insertion, or internal fusion, of therapeutically active peptides, particularly those requiring a secondary structure to be functional, or Therapeutic proteins, to essentially generate an albumin molecule with specific biological activity.

Loops in human albumin structure into which peptides or polypeptides may be inserted to generate albumin fusion proteins of the invention include: Val54-Asn61, Thr76-Asp89, Ala92-Glu100, Gln170-Ala176, His247-Glu252, Glu266-Glu277, Glu280-His288, Ala362-Glu368, Lys439-Pro447,Val462-Lys475, Thr478-Pro486, and Lys560-Thr566. In other embodiments, peptides or polypeptides are inserted into the

Val54-Asn61, Gln170-Ala176, and/or Lys560-Thr566 loops of mature human albumin (WO '480 SEQUENCE).

Peptides to be inserted may be derived from either phage display or synthetic peptide libraries screened for specific biological activity or from the active portions of a molecule with the desired function. Additionally, random peptide libraries may be generated within particular loops or by insertions of randomized peptides into particular loops of the HA molecule and in which all possible combinations of amino acids are represented.

Such library(s) could be generated on HA or domain fragments of HA by one of the following methods:

(a) randomized mutation of amino acids within one or more peptide loops of HA or HA domain fragments. Either one, more or all the residues within a loop could be mutated in this manner;

(b) replacement of, or insertion into one or more loops of HA or HA domain fragments (*i.e.*, internal fusion) of a randomized peptide(s) of length X_n (where X is an amino acid and n is the number of residues;

(c) N-, C- or N- and C- terminal peptide/protein fusions in addition to (a) and/or (b).

The HA or HA domain fragment may also be made multifunctional by grafting the peptides derived from different screens of different loops against different targets into the same HA or HA domain fragment.

Peptides inserted into a loop of human serum albumin are CNTF peptides or peptide fragments or peptide variants thereof. More particularly, the invention encompasses albumin fusion proteins which comprise peptide fragments or peptide variants at least 7 at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 20, at least 25, at least 30, at least 35, or at least 40 amino acids in length inserted into a loop of human serum albumin. The invention also encompasses albumin fusion proteins which comprise peptide fragments or peptide variants at least 7 at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 20, at least 25, at least 30, at least 35, or at least 40 amino acids fused to the N-terminus of human serum albumin. The invention

also encompasses albumin fusion proteins which comprise peptide fragments or peptide variants at least 7 at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 20, at least 25, at least 30, at least 35, or at least 40 amino acids fused to the C-terminus of human serum albumin.

Generally, the albumin fusion proteins of the invention may have one HA-derived region and one CNTF protein-derived region. Multiple regions of each protein, however, may be used to make an albumin fusion protein of the invention. Similarly, more than one CNTF may be used to make an albumin fusion protein of the invention. For instance, a CNTF may be fused to both the N- and C-terminal ends of the HA. In such a configuration, the CNTF portions may be the same or different CNTF molecules. The structure of bifunctional albumin fusion proteins may be represented as: X-HA-Y or Y-HA-X or X-Y-HA or HA-X-Y or HA-Y-X-HA or HA-X-X-HA or HA Y-Y-HA or HA-X-HA-Y or X-HA-Y-HA or multiple combinations and/or inserting X and/or Y within the HA sequence at any location.

Bi- or multi-functional albumin fusion proteins may be prepared in various ratios depending on function, half-life etc.

Bi- or multi-functional albumin fusion proteins may also be prepared to target the CNTF portion of a fusion to a target organ or cell type via protein or peptide at the opposite terminus of HA.

As an alternative to the fusion of known therapeutic molecules, the peptides could be obtained by screening libraries constructed as fusions to the N-, C- or N- and C- termini of HA, or domain fragment of HA, of typically 6, 8, 12, 20 or 25 or X_n (where X is an amino acid (aa) and n equals the number of residues) randomized amino acids, and in which all possible combinations of amino acids were represented. A particular advantage of this approach is that the peptides may be selected *in situ* on the HA molecule and the properties of the peptide would therefore be as selected for rather than, potentially, modified as might be the case for a peptide derived by any other method then being attached to HA.

Additionally, the albumin fusion proteins of the invention may include a linker peptide between the fused portions to provide greater physical separation between the moieties and thus maximize the accessibility of the CNTF portion, for instance, for

binding to its cognate receptor. The linker peptide may consist of amino acids such that it is flexible or more rigid.

Therefore, as described above, the albumin fusion proteins of the invention may have the following formula R2-R1; R1-R2; R2-R1-R2; R2-L-R1-L-R2; R1-L-R2 R2-L-R1; or R1-L-R2-L-R1, wherein R1 is at least one Therapeutic protein, peptide o polypeptide sequence (including fragments or variants thereof), and not necessarily the same Therapeutic protein, L is a linker and R2 is a serum albumin sequence (including fragments or variants thereof). Exemplary linkers include $(GGGGS)_N$ (SEQ ID NO:8)or $(GGGS)_N$ (SEQ ID NO:9) or $(GGS)_N$, wherein N is an integer greater than or equal to 1 and wherein G represents glycine and S represents serine. When R1 is two or more Therapeutic proteins, peptides or polypeptide sequence, these sequences may optionally be connected by a linker.

In further embodiments, albumin fusion proteins of the invention comprising a CNTF protein have extended shelf-life or *in vivo* half-life or therapeutic activity compared to the shelf-life or *in vivo* half-life or therapeutic activity of the same CNTF when not fused to albumin. Shelf-life typically refers to the time period over which the therapeutic activity of a CNTF protein in solution or in some other storage formulation, is stable without undue loss of therapeutic activity. Many of the CNTF proteins are highly labile in their non-fused state. As described below, the typical shelf-life of these CNTF proteins is markedly prolonged upon incorporation into the albumin fusion protein of the invention.

Albumin fusion proteins of the invention with "prolonged" or "extended" shelf-life exhibit greater therapeutic activity relative to a standard that has been subjected to the same storage and handling conditions. The standard may be the non-fused full-length CNTF protein. When the CNTF portion of the albumin fusion protein is an analogue, a variant, or is otherwise altered or does not include the complete sequence for that protein, the prolongation of therapeutic activity may alternatively be compared to the non-fused equivalent of that analogue, variant, altered peptide or incomplete sequence. As an example, an albumin fusion protein of the invention may retain greater than about 100% of the therapeutic activity, or greater than about 105%, 110%, 120%, 130%, 150% or 200% of the therapeutic activity of a standard

when subjected to the same storage and handling conditions as the standard when compared at a given time point. However, it is noted that the therapeutic activity depends on the CNTF protein´s stability, and may be below 100%.

Shelf-life may also be assessed in terms of therapeutic activity remaining after storage, normalized to therapeutic activity when storage began. Albumin fusion proteins of the invention with prolonged or extended shelf-life as exhibited by prolonged or extended therapeutic activity may retain greater than about 50% of the therapeutic activity, about 60%, 70%, 80%, or 90% or more of the therapeutic activity of the equivalent non-fused CNTF when subjected to the same conditions.

Example 1

Preparation of albumin-fused AXOKINE®

CNTF was cloned from human genomic DNA by amplification of the two exons using primers 5´-CTCGGTACCCAGCTGACTTGTTTCCTGG-3´ and 5´-ATAGGATTCCGTAAGAGCAGTCAG-3´ for exon 1, and primer 5´-GTGAAGCATCAGGGCCTGAAC-3´ and 5´-CTCTCTAGAAGCAA GGAAGAGAGAAGGGAC-3´ for exon 2, respectively using standard conditions. Both fragments were ligated under standard conditions, before being re-amplified by PCR using primers 5´-CTCGGTACCCAGCTGACTTGTTTCCTGG-3´ and 5´-CTCTCTAGAAGCAAGGAAGAGAGAAGGGAC-3´ and cloned into vector pCR4 (Invitrogen). To generate AXOKINE® as disclosed in Lambert et al. (PNAS 98:4652-4657; 2001) site-directed mutagenesis was employed to introduce C17A (TGT -> GCT) and Q63R (CAG -> AGA) mutations. DNA sequencing also revealed the presence of a silent T -> C substitution V85V (GTT -> GTC).

To create the C-terminal rHA-GS- AXOKINE®fusion the AXOKINE®cDNA was ligated to a cDNA encoding human albumin by mutagenic PCR using single stranded oligonucleotide primers MH32 5'-TGCCAAGCTTATTACCCAGTCTGATGAGAA GAAATGAAACGAAGGTCATGG-3' and MH35 5'-TGGTGGATCCGGTGGTGC

TTTCACAGAGCATTCACCGCTGACCCC-3' so as to introduce a 14 amino acid GS (-Gly-Gly-Ser-Gly-Gly-Ser-Gly-Gly-Ser-Gly-Gly-Ser-Gly-Gly-) peptide spacer. The amino acid sequence of the mature rHA-GS- AXOKINE® fusion is given in Figure 8.

To create the C-terminal rHA-3xFLAG- AXOKINE® (cleavable AXOKINE® fusion the AXOKINE®cDNA was ligated to a cDNA encoding human albumin by mutagenic PCR using single stranded oligonucleotide primers MH32 5' TGCCAAGCTTATTACCCAGTCTGATGAGA AGAAATGAAACGAAGGTCATGG-3' and CF83 5'-TCATGATATCGA TTACAAGGATGACGATGACAAGGCTTTCACAGAGCATTCACCGCTGACCCCTCA CCGTCGGGACCTCG-3' so as to introduce a 22 amino acid 3xFLAG (-Asp-Tyr-Lys-Asp-His-Asp-Gly-Asp-Tyr-Lys-Asp-His-Asp-Ile-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-) peptide spacer (Sigma-Aldrich Company Ltd.) between the albumin and AXOKINE®sequences. The amino acid sequence of the mature C-terminal rHA-3xFLAG- AXOKINE®fusion is given in Figure 9. The HSA/*MF*□*-1* fusion secretion leader sequence disclosed in WO 90/01063 was provided to ensure secretion of the fusion protein.

To create the N-terminal AXOKINE®-GS-rHA fusion the AXOKINE®cDNA was ligated to a cDNA encoding human albumin by mutagenic PCR using single stranded oligonucleotide primers MH33 5'-ATGCAGATCTTTGGATAAGAGAGCTTT CACAGAGCATTCACCGCTGACCCC-3' and MH36 5'-CACCGGATCCACC CCCAGTCTGATGAGAAGAAATGAAACGAAGGTCATGG-3'so as to introduce either a 14 amino acid GS (-Gly-Gly-Ser-Gly-Gly-Ser-Gly-Gly-Ser-Gly-Gly-Ser-Gly-Gly-) peptide spacer between the AXOKINE®and albumin sequences. The amino acid sequence of the mature AXOKINE-GS-rHA fusion is given in Figure 10.

Maps of the rHA-GS- AXOKINE®sequence, the rHA-3xFLAG-AXOKINE®sequence and the AXOKINE®-GS-rHA sequence are shown in Figures 11, 12 and 13, respectively.

The yeast *PRB1* promoter and the yeast *ADH1* terminator provided appropriate transcription promoter and transcription terminator sequences, respectively as previously disclosed in WO 00/44772 and described by Sleep, D., *et*

al. (1991) Bio/Technology 9, 183-187. Appropriate vector sequences were provide b a "disintegration" plasmid pSAC35 generally disclosed in EP-A-286 424 and describe by Sleep, D., *et al.* (1991) Bio/Technology 9, 183-187.

The rHA fusions were expressed and shake flask culture expression leve determined.

Example 2

Purification

The C-Terminal AXOKINE®contained high levels of clipped material. It was purified using the standard rHA SP-FF conditions (See US Patent No. 6,034,221) bu in a negative mode whereby the fusion was in the flow through. The flow through was adjusted to pH8 and 2.5mS.cm^{-1} and loaded on a standard rHA DE-FF equilibrated in 15mM potassium tetraborate. As for the SP-FF the DEFF was operated in a negative mode. The conductivity of the DE-FF flow through was increased to 15mS.cm^{-1} and the material then purified using standard rHA DBA chromatography with an extra elution of 50mM octanoate. The eluate was then concentrated and diafiltered against 5mM phosphate pH8.3.

The N-Terminal AXOKINE®contained some clipped material. It was purified using the standard rHA SP-FF conditions but in a negative mode whereby the fusion was in the flow through. The flow through was adjusted to pH 8 and 2.5mS.cm^{-1} and loaded on a standard rHA DE-FF equilibrated in 15mM potassium tetraborate. In this instance, a proportion of the fusion bound and was eluted in the standard elution containing 200mM NaCl. The conductivity of the eluate was reduced to 15mS.cm^{-1} and the material purified using standard rHA DBA chromatography with an extra elution of 50mM octanoate. The eluate was then concentrated and diafiltered against 5mM phosphate pH8.3.

The cleavable AXOKINE®contained high levels of clipped material. It was purified using the standard rHA SP-FF conditions but in a negative mode whereby the fusion was in the flow through. The flow through was adjusted to pH 8 and

2.5mS.cm^{-1} and loaded on a standard rHA DE-FF equilibrated in 15mM potassiun tetraborate. As for the SP-FF this was operated in a negative mode. The conductivity of the flow through was increased to 15mS.cm^{-1} and the material purifiec using standard rHA DBA chromatography with an extra elution of 50mM octanoate The material was then concentrated and diafiltered into cleavage buffer. Cleavage was performed overnight at room temperature and the enterokinase removed using Ekapture gel. The material was then concentrated and diafiltered against 5mM phosphate pH8.3. Wurde hier nicht erneut gereinigt?

Example 3

Pharmacokinetics

Assessing the half-life and bioavailability of N-terminal and C-terminal albumin fused AXOKINE® versus non-fused AXOKINE® and assessing additiona pharmacokinetic parameters of N-terminal and C-terminal albumin-fused AXOKINE® versus non-fused AXOKINE®.

Administration Protocol:

Test article 1: Non-fused AXOKINE®

Application volume: 0.33 mL/kg
Single dose/ route: 10 µg/kg i.v. or s.c.
Frequency: 1 x (t=0)

Test article 2: N-terminal albumin-fused AXOKINE®

Application volume: 0.33 mL/kg
Single dose/ route: 40 µg/kg i.v. or s.c.
Frequency: 1 x (t=0)

Test article 3: C-terminal albumin-fused AXOKINE®

Application volume: 0.33 mL/kg

Single dose/ route: 40 µg/kg i.v. or s.c.

Frequency: 1 x (t=0)

Study design

Table 1

Treatment groups

No.	Treatment	Dose / schedule / route	N (M/F)
1	Cleavable AXOKINE®	10 µg/kg / single injection / i.v.	2 m + 2 f
2	C-term. albumin-fused AXOKINE®	40 µg/kg / single injection / i.v.	2 m + 2 f
3	N-term. albumin-fused AXOKINE®	40 µg/kg / single injection / i.v.	2 m + 2 f
4	Cleavable AXOKINE®	10 µg/kg / single injection / s.c.	2 m + 2 f
5	C-term. albumin-fused AXOKINE®	40 µg/kg / single injection / s.c.	2 m + 2 f
6	N-term. albumin-fused AXOKINE®	40 µg/kg / single injection / s.c.	2 m + 2 f

Experimental animals

Species / Strain: rabbits

Sex/Age: 12 males, 12 females; 3-4 months

No. total: 24

Supplier: Fa. Bauer (Neuenstein-Lohe, Germany)

Animal model

Two male and two female rabbits per group received cleavable AXOKINE® (1 µg/kg), C-terminal albumin-fused AXOKINE® (40 µg/kg), or N-terminal albumin-fuse AXOKINE® (40 µg/kg) by a single i.v. or s.c. injection on day 0. Blood samples wer drawn for the determination of the respective antigen levels at baseline and at 5 mir 10 min, 20 min, 30 min, 45 min, 1 h, 2 h, 4 h, 8 h, 24 h (1 d), 48 h (2 d), 72 h (3 d), d, 7 d, 9 d, 11 d, and 14 d after i.v. administration of the respective test substanc and at baseline, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h (1 d), 48 h (2 d), 72 h (3 d), 5 d, 7 d, d, 11 d and 14 d following s.c. injection. Plasma levels of AXOKINE® and albumin fused AXOKINE® were determined by ELISA.

Pharmacokinetic (PK) variables:

Elimination half-life, area under the plasma concentration time curve up tc Day 14 (AUC_{0-14}), maximum concentration (C_{max}). Area under the concentration time curve extrapolated to infinity ($AUC_{0-\infty}$), time of maximum concentration (t_{max}), mear residence time, half-lives of absorption and distribution (if applicable), clearance volume of distribution.

Analytical methods

ELISA determination of non-fused AXOKINE® plasma concentration was performed using a monoclonal mouse anti-hu CNTF-antibody (R&D Systems, clone no. 21809.111) in combination with a biotinylated polyclonal goat anti-hu CNTF antibody (R&D Systems, cat. no. BAF257). Human CNTF was used as standard according to the ELISA kit description.

ELISA determination of albumin-fused AXOKINE® plasma concentration was performed using a monoclonal anti-hu albumin antibody (Aventis Behring GmbH, Laboratory) in combination with a biotinylated polyclonal goat anti-hu CNTF antibody (R&D Systems, cat. no. BAF257). The respective albumin-fused AXOKINE® served for generation of the standard curve.

Using the commercially available human CNTF ELISA (R&D Systems), it wa not possible to detect the albumin-fused AXOKINE®, probably due to sterica interference of the albumin with the binding of the anti-CNTF antibodies.

As a solution, an internal anti-albumin assay was established, using an anti albumin monoclonal antibody as capture antibody, where this antibody was couple to the plate. As the next step, the commercially available CNTF antibody from R&I Systems was used as detection antibody for albumin-fused AXOKINE®.

Analysis of individual plasma levels

The plasma concentration-time profiles of C- and N-terminal albumin-fuse AXOKINE® and non-fused AXOKINE® were analyzed per animal by means o nonlinear regression. An exponential model was fitted to the data by the method o least squares. For the profiles following i.v. administration, an open two-compartmen model was used. For the profiles following s.c. administration, an open one compartment model with first-order input and lag time was used. For the i.v. model, a weighting factor of $1/(\text{predicted concentration})^2$ was applied.

The AUC was calculated a) using the linear trapezoidal rule up to the las measured value (AUC_{0-14}) and b) completing AUC_{0-14} by extrapolation for the perioc between Day 14 and infinity ($AUC_{0-\infty}$).

Summary and comparative analyses

Individual PK results were summarized descriptively per treatment and route of application (minimum, median, maximum, mean, standard deviation).

A two-way analysis of variance was carried out for elimination half-life, AUC and C_{max} (all log-transformed). Fixed factors were sex and treatment group. Appropriate contrasts between treatment groups were evaluated. The possibility of unequal variances was also taken into account.

For the purpose of this analysis it was assumed that ln (half-life), ln (AUC) and ln (C_{max}) each follow a normal distribution.

Elimination half-lives were compared between substances, bioavailabilities in terms of AUC and C_{max} were compared between the routes of administration for the

albumin-fused AXOKINE® groups at an alpha level of 0.1 using two-sided 90% confidence intervals.

Results

The means and standard deviations of the AXOKINE® concentrations at every time point are shown in Figure 1 for the i.v. treated non-fused AXOKINE® group, in Figure 2 for the i.v. treated albumin-fused AXOKINE® groups, and in Figure 3 for the s.c. treated albumin-fused AXOKINE® groups. For the s.c. treated non-fused AXOKINE® group, no concentrations could be measured.

In the animals treated intravenously with non-fused AXOKINE®, the levels fell below 1 pg/mL 4 hours after injection. In the albumin-fused AXOKINE® groups, the levels stayed above 1 ng/mL for 7 days. In the animals treated subcutaneously with the albumin-fused AXOKINE® products, the levels reached their peaks after about 1 day and stayed above 1 ng/mL for 7 days. The pharmacokinetic results are presented in Table 2 for the i.v. treated groups and in Table 3 for the s.c. treated groups. The results for non-fused AXOKINE® were converted to the same units as the albumin-fused AXOKINE® groups, but, with the exception of half-lives and mean residence times, cannot be compared with these because of the different assay methods.

Table 2

Pharmacokinetic results following i.v. administration

		Non-fused AXOKINE®	C-terminal albumin-fused AXOKINE®	N-terminal albumin-fused AXOKINE®
	N	4	4	4
Initital half-life (hr)	Mean	0.11	2.32	7.08
	Std Dev	0.02	1.14	1.84
	Median	0.11	2.38	6.55
	Range	0.09 – 0.13	0.88 – 3.66	5.50 – 9.74
Terminal half-life (hr)	Mean	0.49	36.2	23.5
	Std Dev	0.19	15.4	10.7
	Median	0.50	36.7	19.9
	Range	0.30 – 0.66	17.5 – 54.0	15.1 – 39.1
Mean residence time (hr)	Mean	0.44	43.7	17.0
	Std Dev	0.10	17.6	2.7
	Median	0.45	42.9	15.9
	Range	0.34 – 0.55	23.2 – 65.7	15.4 – 21.0
AUC_{0-14} = $AUC_{0-\infty}$ (hr·ng/mL)	Mean	0.97	4,628	8,389
	Std Dev	0.54	413	1,693
	Median	0.93	4,600	8,394
	Range	0.44 – 1.59	4,272 – 5,039	6,475 – 10,293
	Geom. mean	0.85	4,614	8,259
	SF *	1.84	1.09	1.23
C_{max} (ng/mL)	Mean	2.77	362	820
	Std Dev	1.45	94	178
	Median	2.68	356	803
	Range	1.35 – 4.35	275 – 463	632 – 1,041
Total clearance (mL/hr/kg)	Mean	11,774	2.06	1.18
	Std Dev	6,709	0.33	0.24
	Median	10,735	2.10	1.13
	Range	5,629 – 19,995	1.68 – 2.36	0.95 – 1.51
Total volume of distribution (mL/kg)	Mean	4,745	87.4	20.0
	Std Dev	1,728	30.0	4.5
	Median	4,563	85.7	20.3
	Range	3,089 – 6,762	53.3 – 124.8	15.1 – 24.5

** SF = scatter factor = exp[standard deviation (log-transformed values)]*

Table 3

Pharmacokinetic results following s.c. administration

		C-terminal albumin-fused AXOKINE®	N-terminal albumin-fused AXOKINE®
	N	4	4
Lag time (hr)	Mean	1.75	3.47
	Std Dev	0.75	3.78
	Median	1.54	3.42
	Range	1.11 – 2.80	0.00 – 7.05
Absorption half-life (hr)	Mean	13.3	6.01
	Std Dev	5.5	5.52
	Median	10.9	5.03
	Range	9.9 – 21.4	1.35 – 12.64
Terminal half-life (hr)	Mean	30.5	15.4
	Std Dev	6.8	1.7
	Median	31.8	15.9
	Range	21.3 – 37.4	13.0 – 16.9
AUC_{0-14} = $AUC_{0-\infty}$ (hr·ng/mL)	Mean	3,534	1,986
	Std Dev	383	610
	Median	3,598	1,916
	Range	3,011 – 3,931	1,323 – 2,788
	Geom. mean	3,518	1,917
	SF *	1.12	1.36
C_{max} (ng/mL)	Mean	44.6	45.5
	Std Dev	5.5	14.2
	Median	45.6	45.2
	Range	37.3 – 50.1	28.5 – 63.0
t_{max} (hr)	Mean	24	20
	Std Dev	0	8
	Median	24	24
	Range	24 – 24	8 - 24
Relative total clearance (mL/hr/kg)	Mean	2.75	5.23
	Std Dev	0.33	1.66
	Median	2.68	4.93
	Range	2.42 – 3.22	3.54 – 7.50
Relative total volume of distribution (mL/kg)	Mean	118.8	113.6
	Std Dev	14.0	25.0
	Median	122.9	115.2
	Range	98.6 – 130.7	83.4 – 140.5

*SF = scatter factor = exp[standard deviation (log-transformed values)]

Table 4 shows the results of the analyses of variance regarding the elimination half-life. The differences between non-fused and albumin-fused AXOKINE® following i.v. injection were highly significant. The sex of the animals did not have a significan influence on the half-life.

Table 4

Comparison of elimination half-lives between substances

Route	Parameter	Elimination half-life
i.v.	Estimated ratio (C-terminal albumin-fused AXOKINE® / cleavable AXOKINE®)	72.4
	90% confidence limits	38.5 – 136.4
i.v.	Estimated ratio (N-terminal albumin-fused AXOKINE® / cleavable AXOKINE®)	47.5
	90% confidence limits	24.4 – 92.8

Table 5 shows the results of the analyses of variance regarding the absolute bioavailability. For both albumin-fused products, the differences between the two routes of application were not statistically significant with respect to elimination half-life. The differences regarding AUC and C_{max} were highly significant.

Table 5

Comparison of bioavailabilities between routes of application

Substance	Parameter	Elimination half-life	AUC_{0-14}	C_{max}
C-terminal albumin-fused AXOKINE®	Estimated ratio (s.c. / i.v.)	0.89	0.76	0.13
	90% confidence limits	0.52 – 1.55	0.69 – 0.84	0.10 – 0.16
N-terminal albumin-fused AXOKINE®	Estimated ratio (s.c. / i.v.)	0.70	0.23	0.05
	90% confidence limits	0.42 – 1.17	0.15 – 0.37	0.03 – 0.11

The values for area under the curve and maximum plasma levels of non-fused AXOKINE® cannot be compared directly to those of N-and C-terminal albumin-fused AXOKINE®. In contrast to this, the comparison of the half-lives is valid.

Both albumin-fused AXOKINE® preparations showed a markedly prolonged elimination from plasma after i.v. application compared to non-fused AXOKINE®. C-terminal albumin-fused AXOKINE® showed an average elimination half-life that was 72 times longer than that of non-fused AXOKINE®. N-terminal albumin-fused AXOKINE® showed an average elimination half-life that was 48 times longer than that of non-fused AXOKINE®.

In terms of AUC, the absolute bioavailability after s.c. injection was 76% for C-terminal albumin-fused AXOKINE® and 23% for N-terminal albumin-fused AXOKINE®. Since plasma levels of non-fused AXOKINE® were below the detection limit after s.c. application, the comparison with the i.v. application could not be made.

Example 4

Pharmacodynamics

The purpose of this example was to assess the efficacy of N- and C-termina albumin-fused AXOKINE® as compared to placebo or non-fused AXOKINE® in reduction of body weight in leptin-deficient or dietary-induced obese mice.

Study Design of pharmacodynamic animal study, part I

This study was designed as a randomised, partly blinded, parallel, 13-armed trial with two experimental settings (leptin-deficiency induced obesity versus dietary induced obesity) including a total of 70 female C57BL/6Jlep^{ob} (ob/ob), and 41 male and 41 female C57BL/6J mice.

Experimental Animals

C57BL/6Jlep^{ob} (ob/ob) mice were fed standard diet for approximately 3 months. During this time, C57BL/6Jlep^{ob} (ob/ob) mice strongly increased weight due to uncontrolled food intake associated with leptin-deficiency. In wild-type C57BL/6 mice, obesity was induced by feeding with high caloric food containing 45 % of fat. Body weight was recorded weekly during this phase of obesity induction preceding therapeutic treatment. After a mean weight increase to at least 130 % of baseline, treatment with the test substances was started. Test substances (Non-fused AXOKINE®, albumin-fused AXOKINE®, placebo) were administered by daily subcutaneous injections over a period of seven days. During the treatment phase, body weights were determined daily. The mean weight loss as compared to baseline and placebo was calculated to assess the relative efficacy of the test substances.

Study Medication and Dosage

Test article 1:	Placebo (5mM phosphate buffer at pH 8.3)
Endotoxin content:	0.007 EU/mL
Stock concentration:	n.a.
Application volume:	250 µl[a]

Single dose/ route:	n.a. / s.c.
Frequency:	seven daily injections
Test article 2:	Non-fused AXOKINE®
Endotoxin content:	14.9 EU/m2L
Stock concentration:	0.1 mg/mL
Application volume:	250 µl[a]
Single dose/ route:	according to table 1 & 2/ s.c.
Frequency:	seven daily injections
Test article 3:	N-terminal albumin-fused AXOKINE®
Endotoxin content:	1.8 EU/mL
Stock concentration:	5 mg/mL
Application volume:	250 µl[a]
Single dose/ route:	according to table 1 & 2/ s.c.
Frequency:	seven daily injections
Test article 4:	C-terminal albumin-fused AXOKINE®
Endotoxin content:	64 EU/mL & 32 EU/mL
Stock concentration:	0.2 mg/mL
Application volume:	250 µl[a]
Single dose/ route:	according to table 1 & 2/ s.c.
Frequency:	seven daily injections

[a]All mice received 250 µl test substance at treatment day 1 (Day 83), then, dosing was adjusted to body weight changes by adjustment of the administered volume Mice in group 13 (1200 µg/kg C-terminal AXOKINE®) received approximately 390 µ at Day 83.

Table 6 Treatment groups C57BL/6Jlep^{ob} (ob/ob) mice

No.	Treatment	Dose / volume / schedule /route	N (m/f
1	Placebo	- / 250 µl/ 7 daily injections / s.c.	10 f
2	Non-fused AXOKINE®	10 µg/kg / 250 µl / 7 daily injections / s.c.	5 f
3	Non-fused AXOKINE®	100 µg/kg / 250 µl / 7 daily injections / s.c.	5 f
4	Non-fused AXOKINE®	300 µg/kg on Days 1-2, 200 µ/kg on treatment days 3-7 / 250 µl / 7 daily injections / s.c.	5 f
5	N-albumin-fused AXOKINE®	40 µg/kg /250 µl / 7 daily injections / s.c.	5 f
6	N-albumin-fused AXOKINE®	280 µg/kg on Days 1-2, 200 µ/kg on treatment days 3-7 / 250 µl / 7 daily injections / s.c.	5 f
7	N-albumin-fused AXOKINE®	400 µg/kg / 250 µl / 7 daily injections / s.c.	5 f
8	N-albumin-fused AXOKINE®	1200 µg/kg on Days 1-2, 800 µ/kg on treatment days 3-7 / 250 µl / 7 daily injections / s.c.	5f
9	C-albumin-fused AXOKINE®	40 µg/kg / 250 µl / 7 daily injections / s.c.	5 f
10	C-albumin-fused AXOKINE®	280 µg/kg on Days 1-2, 200 µ/kg on treatment days 3-7 / 250 µl / 7 daily injections / s.c.	5 f
11	C-albumin-fused AXOKINE®	400 µg/kg / 250 µl / 7 daily injections / s.c.	5 f
12	C-albumin-fused AXOKINE®	1200 µg/kg on Days 1-2, 800 µ/kg on treatment days 3-7 / 390-250 µl / 7 daily injections / s.c.	5 f

Table 7 Treatment groups C57BL/6J mice

No.	Treatment	Dose / volume / schedule /route	N (m/f)
1	Placebo	- / 250 µl/ 7 daily injections / s.c.	5m/5f
2	Non-fused AXOKINE®	10 µg/kg / 250 µl / 7 daily injections / s.c.	3m/3f
3	Non-fused AXOKINE®	100 µg/kg / 250 µl / 7 daily injections / s.c.	3m/3f
4	Non-fused AXOKINE®	300 µg/kg on Days 1-2, 200 µg/kg on treatment days 3-7 / 250 µl / 7 daily injections / s.c.	3m/3f
5	N-albumin-fused AXOKINE®	40 µg/kg /250 µl / 7 daily injections / s.c.	3m/3f
6	N-albumin-fused AXOKINE®	280 µg/kg on Days 1-2, 200 µg/kg on treatment days 3-7 / 250 µl / 7 daily injections / s.c.	3m/3f
7	N-albumin-fused AXOKINE®	400 µg/kg / 250 µl / 7 daily injections / s.c.	3m/3f
8	N-albumin-fused AXOKINE®	1200 µg/kg on Days 1-2, 800 µg/kg on treatment days 3-7 / 250 µl / 7 daily injections / s.c.	3m/3f
9	C-albumin-fused AXOKINE®	40 µg/kg / 250 µl / 7 daily injections / s.c.	3m/3f
10	C-albumin-fused AXOKINE®	280 µg/kg on Days 1-2, 200 µg/kg on treatment days 3-7 / 250 µl / 7 daily injections / s.c.	3m/3f
11	C-albumin-fused AXOKINE®	400 µg/kg / 250 µl / 7 daily injections / s.c.	3m/3f
12	C-albumin-fused AXOKINE®	1200 µg/kg on Days 1-2, 800 µg/kg on treatment days 3-7 / 390-250 µl / 7 daily injections / s.c.	3m/3f

The following dose reductions had to be made for both, ob/ob as well as wildtype mice:

Non-fused AXOKINE® from Delta: from 300µg/kg on Day 1-2 to 200µg/kg on Day 3-7

N, C-terminal AXOKINE®: from 280µg/kg on Day 1-2 to 200µg/kg on Day 3-7

N, C-terminal AXOKINE®: from 1200µg/kg on Day 1-2 to 800µg/kg on Day 3-7

Randomisation was done according to the randomisation list, separately fo C57BL/6Jlep^{ob} (ob/ob) and for C57BL/6J mice. After the mice were randomised tc cages, cages were randomised to treatment.

Efficacy variables: Bodyweight (determined daily from Day 0 –7).

Analytical Methods

Body weights were recorded by weighing of conscious animals.

Statistical Methods

Primary efficacy variable: Body weight difference between Day 7 and Day 0 and up to Day 102. Separated for C57BL/6Jlep^{ob} (ob/ob) and for C57BL/6J mice dose-response relationships for non-fused AXOKINE®, N-terminal albumin-fused AXOKINE®, and C-terminal albumin-fused AXOKINE® were analyzed within one analysis of variance model:

Successive comparison of the different doses with placebo using contrasts (e.g. Helmert or reverse Helmert contrasts) in order to identify the minimal effective dose. Comparison of pairs of equimolar doses using 2-sided t-tests and 2-sided 95% confidence intervals for the difference.

An overall assessment of N-terminal albumin-fused AXOKINE® and C-terminal albumin-fused AXOKINE® with regard to nonfused AXOKINE® was done by means of a parallel line assay with log-transformed doses. The derived potency was supplemented by a 95% confidence interval.

Results

Statistical Analysis of primary endpoint

Endpoint: Body weight change (g) from Day 82 to Day 91, 92, 93, 94, 95, 96, 102

Statistics: F-tests within ANOVA in ordered hypotheses families. Starting on Day 92 a hypothesis is rejected provided the corresponding F-test is significant and the preceding hypothesis has also been rejected.

Reference: Bauer P: Multiple tests in clinical trials. Statistics in Medicine, 10:871 890, 1991

Weight Reduction in ob/ob mice

Figures 4, 5, 6 and 7 compare equimolar doses of the non-fused AXOKINE® with albumin fused AXOKINE® in leptin deficient mice.

In summary, the pharmacodynamic data show that in the leptin deficient mice albumin fused AXOKINE® is statistically significant better than the non fused AXOKINE® for dose groups 11, 12, and 13. In wild type mice, the albumin fused AXOKINE® is statistically better compared to the non-fused AXOKINE® in group 12.

Study design of pharmacodynamic animal study, part II

The study was originally designed as a randomized, partly blinded, parallel, 11-armed trial with two experimental settings (leptin-deficiency induced obesity versus dietary-induced obesity) including a total of 82 female B6.V-Lep^{ob} (ob/ob) mice, and 41 male and 41 female C57BL/6J mice. Due to restricted availability of non-fused AXOKINE®, only selected treatment groups of leptin-deficient mice were included in the treatment phase of the study (Table 8).

Table 8: Treatment groups B6.V-Lep^{ob} (ob/ob) mice

No.	Treatment	dose / volume / schedule /route	n (m/f)
1	Placebo	- / 5 µl/g / 7 daily injections / s.c.	10 f
2	Non-fused AXOKINE®	100 µg/kg / 5 µl/g / Days 1, 4, 7 / s.c.	6 f
3	Non-fused AXOKINE®	300 µg/kg / 5 µl/g / Days 1, 4, 7 / s.c.	6 f
4	Non-fused AXOKINE®	100 µg/kg / 5 µl/g / 7 daily injections / s.c.	6 f
5	Non-fused AXOKINE®	300 µg/kg / 5 µl/g / 7 daily injections / s.c.	6 f
6	C-albumin-fused AXOKINE®	400 µg/kg / 5 µl/g / Days 1, 4, 7 / s.c.	6 f
7	C-albumin-fused AXOKINE®	1200 µg/kg / 5 µl/g / Days 1, 4, 7 / s.c.	6 f
8	C-albumin-fused AXOKINE®	3600 µg/kg / **10** µl/g / Days 1, 4, 7 / s.c.	6 f
9	C-albumin-fused AXOKINE®	400 µg/kg / 5 µl/g / 7 daily injections / s.c.	6 f
10	C-albumin-fused AXOKINE®	1200 µg/kg / 5 µl/g / 7 daily injections / s.c.	6 f
11	C-albumin-fused AXOKINE® (stability: 14 days at room temperature)	1200 µg/kg / 5 µl/g / 7 daily injections / s.c.	6 f

Schedule

B6.V-Lep^{ob} mice were fed standard diet until Day 80 and increased weight. Treatment with either non-fused AXOKINE® or C-albumin-fused AXOKINE® started on Day 81 either for seven consecutive days (Days 81, 82, 83, 84, 85, 86, 87) or only on Days 1, 4, 7 (Days 81, 84, 87).

Body weight was assessed until 21 days post-treatment cessation (Day 108). Body weight changes and pertaining analyses were related to the weight on Day 81.

The corresponding timepoints are summarized in the table below:

Table 9 Treatment schedule B6.V-Lep^{ob} (ob/ob) mice

Study Day	*Treatment day*
Day 81	Day 1
Day 84	Day 4
Day 87	Day 7
Day 101	Day 21
Day 108	Day 28

Administration of test articles

Test article 1:	Placebo (5mM phosphate buffer at pH 8.3)
Manufacturer:	Aventis Behring (Laboratory Dr. H.Metzner)
Batch No.:	-
Endotoxin content:	n.t.
Stock concentration:	n.a.
Application volume:	5 µl/g
Single dose/ route:	n.a. / s.c.
Frequency:	seven daily injections
Test article 2:	Non-fused AXOKINE® (Enterokinase-cleaved C-terminal albumin-fused AXOKINE®)
Manufacturer:	Delta Biotechnology Ltd., Laboratory Dr. D. Sleep
Batch No.:	1675#40
Endotoxin content:	18 EU/mL
Stock concentration:	approximately 0.1 mg/mL (assumption based on SDS PAGE with Coomassie staining compared to CNTF as a standard, Appendix B)
Application volume:	5 µl/g
Single dose/ route:	according to table 1 / s.c.
Frequency:	single injections on days 1, 4, 7 or seven daily injections
Test article 3:	C-terminal albumin-fused AXOKINE®
Manufacturer:	Delta Biotechnology Ltd., Laboratory Dr. D. Sleep/ Aventis Behring GmbH, Laboratory Dr. H. Metzner
Batch No.:	091002
Endotoxin content:	16 EU/mL
Stock concentration:	approximately 0.4 mg/mL (assumption based on SDS PAGE with Coomassie staining compared to HSA as a standard, Appendix B)
Application volume:	5 µl/g[a]
Single dose/ route:	according to table 1 /s.c.
Frequency:	single injections on days 1, 4, 7 or seven daily injections

Test article 4:	C-terminal albumin-fused AXOKINE® stored a room temp. for 14 days
Manufacturer:	Delta Biotechnology Ltd., Laboratory Dr. D. Sleep Aventis Behring GmbH, Laboratory Dr. H. Metzner
Batch No.:	091002
Endotoxin content:	16 EU/mL
Stock concentration:	approximately 0.4 mg/mL (assumption based on SDS PAGE with Coomassie staining compared to HSA as a standard, Appendix B)
Application volume:	5 µl/g[a]
Single dose/ route:	according to table 1 /s.c.
Frequency:	single injections on days 1, 4, 7 or seven daily injections

[a]All mice received 5 µl/g test substance except mice treated with C-terminal AXOKINE® 3600 µg/kg which received 10 µl/g.

Animal model

B6.V-Lep^{ob} (ob/ob) mice were fed standard diet for 12 weeks. During this time, mice strongly increased weight due to uncontrolled food intake associated with leptin-deficiency. Body weight was recorded weekly during this phase of obesity induction preceding therapeutic treatment with the exception of days 49-66, when animals were not weighed. Test substances (AXOKINE®, C-terminal albumin-fused AXOKINE®, placebo) were administered either by daily subcutaneous injections over a period of seven days or by three single injections at treatment Days 1, 4, 7. During the treatment phase, body weights were determined daily. Thereafter, body weight was recorded every other working day (i.e. 3 times per week) for 14 days and once more at 21 days post treatment (Day 28 after treatment start = study Day 108). The mean weight loss as compared to baseline and placebo was calculated to assess the relative efficacy of the test substances.

Randomization

Randomization was done according to the randomization list. After randomization of mice to cages, cages were randomized to treatment.

Efficacy Variables

major: Body weight change (g) from treatment Day 1 (study Day 81) to treatment Day 7 (Study Days 88, 87, 86, 85, 84, 83, and 82).

minor: Body weight at Day 28 after start of treatment (Study Day 108). Body weight change (g) from Study Day 81 to Days 89, 91, 94, 96, 98, 101, 108.

Analytical Methods

Body weights were recorded by weighing of conscious animals.

Statistical Methods

F-tests within ANOVA in ordered hypotheses families. Starting on Day 88 and then proceeding downward, a hypothesis was rejected provided the corresponding F-test was significant ($p \leq 0.05$) and the preceding hypotheses had also been rejected ($p \leq 0.05$). The same procedure was applied starting on Day 89 upward until Day 108. The procedure controlleds the multiple level 0.05 within a set of comparisons, which consisted of the seven hypotheses related to the days.

Four blocks of analyses were conducted: Tables 10 and 11 compile test decisions for tests against placebo, i.e. active treatment groups (groups 2-11) were compared with placebo (group 1) in order to check model validity. While analyses of equimolare doses are provided in Tables 12 and 13, treatment schedules are compared in Tables 14 and 15. Finally, potency estimations are summarized in Table 16, using a parallel line assay on log-doses with Day 88 body weight change serving as response criterion. Tests on the suitability (i.e. linearity, parallelism) of the assay approach were not done.

Results

Effects on body weight

Study treatment was administered from Day 81 to Day 87.

Comparisons with placebo

All groups receiving test substances showed a significant difference to placebo between Day 82 and Day 101 (Table 10 and 11).

Table 10: Test decisions for comparison against placebo (i.e. validity of model – Day 82)

Comparison	Day						
	88	87	86	85	84	83	82
2 vs. 1	*	*	*	*	*	*	*
3 vs. 1	*	*	*	*	*	*	*
4 vs. 1	*	*	*	*	*	*	*
5 vs. 1	*	*	*	*	*	*	*
6 vs. 1	*	*	*	*	*	*	*
7 vs. 1	*	*	*	*	*	*	*
8 vs. 1	*	*	*	*	*	*	*
9 vs. 1	*	*	*	*	*	*	*
10 vs. 1	*	*	*	*	*	*	*
11 vs. 1	*	*	*	*	*	*	*

note: * (#) indicates that first (second) group shows a significantly ($p < 0.05$) larger weight reduction than second
(first) group of the comparison.
"–" indicates absence of significance.

Table 11: Test decisions for comparison against placebo (i.e. validity of model) – L 89-108

Comparison	Day						
	89	91	94	96	98	101	108
2 vs. 1	*	*	*	*	*	*	-
3 vs. 1	*	*	*	*	*	*	-
4 vs. 1	*	*	*	*	*	*	*
5 vs. 1	*	*	*	*	*	*	*
6 vs. 1	*	*	*	*	*	*	-
7 vs. 1	*	*	*	*	*	*	*
8 vs. 1	*	*	*	*	*	*	*
9 vs. 1	*	*	*	*	*	*	*
10 vs. 1	*	*	*	*	*	*	*
11 vs. 1	*	*	*	*	*	*	*

note: * (#) indicates that first (second) group shows a significantly ($p < 0.05$) larger weight reduction than second (first) group of the comparison.
"–" indicates absence of significance.

Comparisons of equimolar doses

Table 12: Test decisions for equimolare doses – Days 88-82

1. Comparison	2. Day 88	87	86	85	84	83	82
Day 1, 4, 7 schedule							
2 vs. 6	#	#	#	#	#	–	–
3 vs. 7	#	#	#	#	#	#	#
Day 1-7 schedule							
4 vs. 9	#	#	#	#	–	–	–
5 vs. 10	#	#	#	#	#	–	–
5 vs. 11	#	#	#	#	#	#	–

note: * (#) indicates that first (second) group shows a significantly ($p < 0.05$) large weight reduction than second (first) group of the comparison
"–" indicates absence of significance.

Table 13: Test decisions for equimolare doses – Days 89-108

1. Comparison	2. Day 89	91	94	96	98	101	108
Day 1, 4, 7 schedule							
2 vs. 6	#	#	#	#	–	–	–
3 vs. 7	#	#	#	#	#	#	#
Day 1, 4, 7 schedule							
4 vs. 9	#	#	#	–	–	–	–
5 vs. 10	#	#	#	#	#	–	–
5 vs. 11	#	#	#	#	#	#	#

note: * (#) indicates that first (second) group shows a significantly ($p < 0.05$) larger weight reduction than second
(first) group of the comparison.
"–" indicates absence of significance.

Comparisons of treatment schedules

Table 14: Test decisions for comparison of treatment schedules (Day 1, 4, 7 vs. Day – Day 88-82

Comparison	Day 88	87	86	85	84	83	82
Non-fused AXOKINE®							
2 vs. 4	#	#	#	#	#	#	–
3 vs. 5	#	#	#	#	#	#	–
C-albumin-fused AXOKINE®							
6 vs. 9	#	#	#	#	#	#	–
7 vs. 10	#	#	#	#	#	–	–
7 vs. 11	#	#	#	#	#	#	#

note: * (#) indicates that first (second) group shows a significantly ($p < 0.05$) larger weight reduction than second (first) group of the comparison
"–" indicates absence of significance.

Table 15: Test decisions for comparison of treatment schedules (Day 1, 4, 7 vs. Day – Day 89-108

Comparison	Day 89	91	94	96	98	101	108
Non-fused AXOKINE®							
2 vs. 4	#	#	#	#	#	–	–
3 vs. 5	#	#	#	#	#	#	#
C-albumin-fused AXOKINE®							
6 vs. 9	#	#	#	#	#	#	#
7 vs. 10	#	#	#	#	#	–	–
7 vs. 11	#	#	#	#	#	#	–

note: * (#) indicates that first (second) group shows a significantly ($p < 0.05$) larger weight reduction than second (first) group of the comparison.
"–" indicates absence of significance.

Potency estimation

Table 16: Potency#) estimations using Day 88 body weight change

Comparison	Potency
7 daily injection: albumin-fused AXOKINE®/ AXOKINE®	1.90
Day 1, 4, 7 injection: albumin-fused AXOKINE®/ AXOKINE®	2.33
albumin-fused AXOKINE®: 7 daily/Day 1, 4, 7	9.13
Non fused AXOKINE®: 7 daily/Day 1, 4, 7	1.85
Day 1, 4, 7 injection albumin-fused AXOKINE®/7 daily injections non fused AXOKINE®	0.87

#) Parallel line assay for Day 88 body weight change on log-dose was used Group 1 and 11 were never included into the calculations.

The observed prolongation of the plasma half life of albumin-fused AXOKINE as investigated in rabbits (72 times longer than non-fused AXOKINE) while admistering the fusion protein s.c. is very surprising. First the s.c. administration is known to result in reduced resorption which is not the case here. Second as it is normally known that plasma half lives of human plasma proteins are sometimes dramatically reduced in animals this prolongation points out that the situation in humans is even more pronounced. This is confirmed by our pharmacodynamic findings in mice, where it was possible to adminster the fusion protein every third day with nearly comaprable efficacy compared to the daily application of the non-fused AXOKINE. As a consequence we speculate that it might be possible to administer the fusion protein perhaps in humans in weekly or even longer intervals. Furthermore efficacy and safety might be increased as could be a decreased rate of the generation of antibodies towards CNTF.

Clinical observations

Six Animals were prematurely withdrawn from the study, all after completion of the treatment:

Starting on day 84 all animals of groups 8, 10 and 11 (receiving 1200µg/kg C-AFP daily or 3600 µg/kg C-AFP 3x) showed a dull, ruffled coat, generalized reddening of the skin and reduced general condition. Up to 10 of the 12 animals receiving 1200 µg and all animals treated with 3600 µg developed bloody diarrhea over the following two days, accompanied by reduced water intake, leading to severe dehydration. Therefore one animal in each of the groups 10, 11 on Day 89, three animals in group 8 on Day 91 were euthanized in extremis. One further animal of group 10 died on Day 96.

At necropsy severe obesity, dehydration, and fatty degeneration of liver and kidneys, together with dilated intestines were found in all examined animals.

CONCLUSION

Prior to start of treatment (Day 81) a total of 70 animals were available, 10 animals in the placebo group (group 1), and six animals in each of the 10 active treatment groups. A total of six animals was eutharized or died during the study course, all after completion of the treatment: one animal in each of the groups 10, 11 on Day 89 three animals in group 8 on Day 91, and finally one further animal in group 10 on Day 96. These cases and the observed clinical symptoms were confined to the highest dose groups, and are thus considered as treatment-related.

The weight of placebo treated animals was nearly constant between Day 81 and Day 88 (mean weight change on Day 88: –0.4 %), but in the further course of the trial a weight increase until Day 108 (mean change on Day 108: 7.2 %) was noticed.

Treatment with active substances (groups 2-11) led to significant dose-dependent body weight reductions as compared with placebo (Table 10). Even within 21 days after treatment completion animals treated with an active substance showed significantly higher body weight reductions than placebo animals (Table 11).

When comparing equimolare doses, albumin-fused- AXOKINE® was considerably better than non-fused AXOKINE® with respect to the body weight reduction (Table 12, Figure 14), no matter which treatment schedule was applied. After the end of treatment this effect continued dose-dependently (Table 13), for groups 7, 11 even until Day 108.

Daily injections over seven days resulted in a more pronounced effect than injections on Days 1, 4, 7 (Table 7, 8), for both under therapy and during the 21 follow-up period. This held for the comparisons within non-fused AXOKINE® and within C-albumin fused AXOKINE.

Potency estimations were confined to the body weight change on Day 88. albumin-fused AXOKINE®was 1.9 and 2.3 times more potent than non-fused AXOKINE®for the seven days treatment schedule and the schedule with treatment on Days 1, 4, 7, respectively (Table 16). Treatments on Days 1-7 were more potent than treatments on Day 1, 4, 7. For non-fused AXOKINE®a potency of 1.85 and for the albumin-fused AXOKINE®a potency of 9.13 was calculated.

Injections with albumin-fused AXOKINE®on Day 1, 4, 7 were nearly as potent as daily injections on seven consecutive days with unfused AXOKINE.

List of Cited References

1. Kalra SP, Dube MG, Pu S, Xu B. Horvath TL, Kalra PS. Interacting appetite regulating pathways in the hypothalamic regulation of body weigh Endocrine Reviews 1999; *20*: 68-100.

2. Ahima RS, Osei SY. Molecular regulation of eating behaviour: new insight and prospects for therapeutic strategies TRENDS in Molecular Medicine 2001; *7*: 205-213.

3. Dove A. Biotech weighs up the options in obesity Nature Biotechnology 2001; *19*: 25-28.

4. Van der Ploeg, LHT. Obesity: an epidemic in need of therapeutics Current opinion in Chemical Biology 2000; *4*: 452-460.

5. Wieland HA, Hamilton BS. Weighing the options in the pharmacotherapy of obesity. International Journal of Clinical Pharmacology and Therapeutics 2001; *39*: 406-414.

6. Inui, A. Transgenic study of energy homeostasis equation: implications and confounding influences. The FASEB Journal 2000; *14*: 2158-2170.

7. Lambert PD, Anderson KD, Sleeman MW, Wong V, Tan J, Hijarunguru A Corcoran TL, Murray JD, Thabet KE, Yancopoulos GD, Wiegand SJ Ciliary neurotrophic factor activates leptin-like pathways and reduces body fat without cachexia or rebound weight gain, even in leptin-resistant obesity. PNAS 2001; *98*: 4652-4657.

8. ALS CNTF Treatment Study Group: A double-blind placebo-controlled clinical trial of subcutaneous recombinant human ciliary neurotrophic factor (rHCNTF) in amyotrophic lateral sclerosis. Neurology 1996; *46*: 1244-1249.

9. Kalra SP. Circumventing leptin resistance for weight control. PNAS 2001; *98*: 4279-4281.

10. Podusio JF, Curran GL. Permeability at the blood-brain and blood-nerve barriers of the neurotrophic factors: NGF, CNTF, NT-3, BDNF. Molecular Brain Research 1996; *36*: 280-286.

11. Pan W, Kastin AJ, Maness LM, Brennan JM. Saturable entry of ciliary neurotrophic factor into brain. Neuroscience Letters 1999; *263*: 69-71.

12. Bickel U, Yoshikawa T, Pardridge WM. Delivery of peptides and protein through the blood-brain barrier Advances in Drug Delivery Review 2001; *46*: 247-79

13. Gloor SM, Wachtel M, Bolliger MF, Ishihara H, Landmann R, Frei K Molecular and cellular permeability control at the blood-brain barrier Brain Research Reviews 2001; *36*: 258-264.

14. Guler HP, Acheson A, Stambler N, Hunt TL, Dato M. *Safety study with* AXOKINE® *(Rm): a second generation ciliary neurotrophic factor* International Journal of Obesity and Related Metabolic Disorders. *24* (Suppl 1): 102, May 2000. USA.

15. Guler HP, Ettinger MP, Littlejohn TW, Schwartz SL, Weiss SL, Mcliwain HH Heymsfield SB, Bray GA, Roberts WG, Acheson A, Heyman ER, Dark CL Vicary C. *AXOKINE® causes significant weight loss in severely and morbidly obese subjects.* International Journal of Obesity and Related Metabolic Disorders 2001; 25: S111: P 291.

16. Regeneron Pharmaceuticals Inc. *Regeneron gets positive weight loss results from AXOKINE.* Media Release. : [6 pages], 29 Nov 2000. Available from URL: http://www.regeneron.com. USA.

17. Regeneron Pharmaceuticals Inc. *Regeneron updates phase II obesity trial results; weight loss maintained 36 weeks following treatment cessation.* Media Release: [6 pages], 11 Sep 2001. Available from: URL: http://www.regeneron.com. USA.

18. Regeneron Pharmaceuticals Inc. *Regeneron updates obesity drug results; patients maintained weight loss following treatment cessation.* Media Release. : [4 pages], 28 Feb 2001. Available from: URL: http://www.regeneron.com. USA.

19. SCRIP No 2720 Feb. 2002: *Question over* AXOKINE®*safety.*

Claims:

1. A fusion protein comprising an albumin, or a fragment or a variant or a derivative thereof and at least one biologically active peptide or protein which activates the ciliary neurotrophic factor (CNTF) receptor, or a fragment or variant or a derivative thereof.

2. The fusion protein of claim 1, wherein the at least one peptide or protein which activates the ciliary neurotrophic factor (CNTF) receptor is CNTF or a fragment or variant or a derivative thereof.

3. The fusion protein of claim 2, wherein the CNTF is AXOKINE®

4. The fusion protein of any of claims 1 to 3 wherein the in-vivo half-life of the fusion protein is greater than the in-vivo half-life of the unfused biologically active peptide or protein

5. The fusion protein of any of claims 1 to 3 wherein the shelf-life of the fusion protein is greater than the shelf-life of the unfused biologically active peptide or protein.

6. The fusion protein of any of claims 1 to 5 which is expressed in yeast.

7. The fusion protein of any of claims 1 to 5 which is expressed in a mammalian cell.

8. The fusion protein of any of claims 1 to 5 wherein the mammalian cell is human cell.

9. A pharmaceutical composition comprising an effective amount of the fusio protein of any of claims 1 to 8 and a pharmaceutically acceptable carrier c excipient.

10. The use of a fusion protein of any of claims 1 to 8 for the manufacture of a medicament for treating obesity and diseases associated therewith.

11. The use according to claim 10, wherein the disease associated with obesity is diabetes, hyperglycemia or hyperinsulinemia.

12. A method for extending the half-life of a biologically active peptide or protein which activates the ciliary neurotrophic factor (CNTF) receptor, or a fragmen or variant or a derivative thereof in a mammal, the method comprising linking said biologically active peptide or protein to an albumin to form an albumin-fused biologically active peptide or protein and administering said albumin-fused biologically active peptide or protein to said mammal, whereby the half-life of said albumin-fused biologically active peptide or protein is extended a least 2-fold over the half-life of the biologically active peptide or protein lacking the linked albumin.

13. The method of claim 12, wherein the biologically active peptide or protein is CNTF or a fragment or variant or a derivative thereof.

14. The method of any of claims 12 to 13 , wherein the half-life of said albumin-fused biologically active peptide or protein is extended at least 5-fold over the half-life of the biologically active peptide or protein lacking the linked albumin.

15. The method of any of claims 12 to 13, wherein the half-life of said albumir fused biologically active peptide or protein is extended at least 10-fold ove the half-life of the biologically active peptide or protein lacking the linke albumin.

16. The method of any of claims 12 to 13 , wherein the half-life of said albumin fused biologically active peptide or protein is extended at least 50-fold ove the half-life of the biologically active peptide or protein lacking the linke albumin.

17. A method for increasing the concentration of a biologically active peptide o protein across the blood brain barrier, the method comprising linking saic biologically active peptide or protein to an albumin to form an albumin-fusec biologically active peptide or protein and administering said albumin-fusec biologically active peptide or protein to said mammal, whereby the concentration of said albumin-fused biologically active peptide or protein is increased across the blood brain barrier over the concentration of the biologically active peptide or protein lacking the linked albumin.

18. The method of claim 17, wherein the biologically active peptide or protein activates the ciliary neurotrophic factor (CNTF) receptor, or is a fragment or variant or a derivative thereof.

19. The method of claim 17, wherein the biologically active peptide or protein is CNTF or a fragment or variant or a derivative thereof.

20. A method for minimizing side effects associated with the treatment of a mammal with a biologically active peptide or protein activates the ciliary

neurotrophic factor (CNTF) receptor, or a fragment or variant or a derivativ thereof, the method comprising linking said biologically active peptide c protein to an albumin to form an albumin-fused biologically active peptide c protein and administering said albumin-fused biologically active peptide c protein to said mammal.

21. The method of claim 20, wherein the biologically active peptide or protei activates the ciliary neurotrophic factor (CNTF) receptor, or is a fragment o variant or a derivative thereof.

22. The method of claim 20, wherein the biologically active peptide or protein is CNTF or a fragment or variant or a derivative thereof.

23. The method of any claims 20 to 22, wherein said side effect is nausea and/ or headache.

24. A nucleic acid molecule comprising a polynucleotide sequence encoding for a fusion protein according to any of the claims 1 to 8.

25. A vector comprising the nucleic acid molecule of claim 24.

25. A host cell containing the nucleic acid molecule of claim 24.

26. A method of activating the CNTF-receptor in a cell, which method comprises the step of contacting said cell with an effective concentration of a fusion protein according to any of claims 1 to 8.

27. The method of claim 26, wherein the cell is a mammalian cell.

28. The method of claim 27, wherein the cell is a human cell.

29. A method of activating the CNTF-receptor in a cell, which method comprises the step of providing said cell with an effective concentration of a fusion protein according to any of claims 1 to 8, by introducing a nucleic acid molecule according to claim 24 into the cell, enabling said cell to produce a therapeutically effective amount of a fusion protein according to any of claims 1 to 8.

30. The method of claim 29, wherein the cell is a mammalian cell.

31. The method of claim 30, wherein the cell is a human cell.

Figure 1: Mean Axokine concentrations +/− SD, following i.v. application

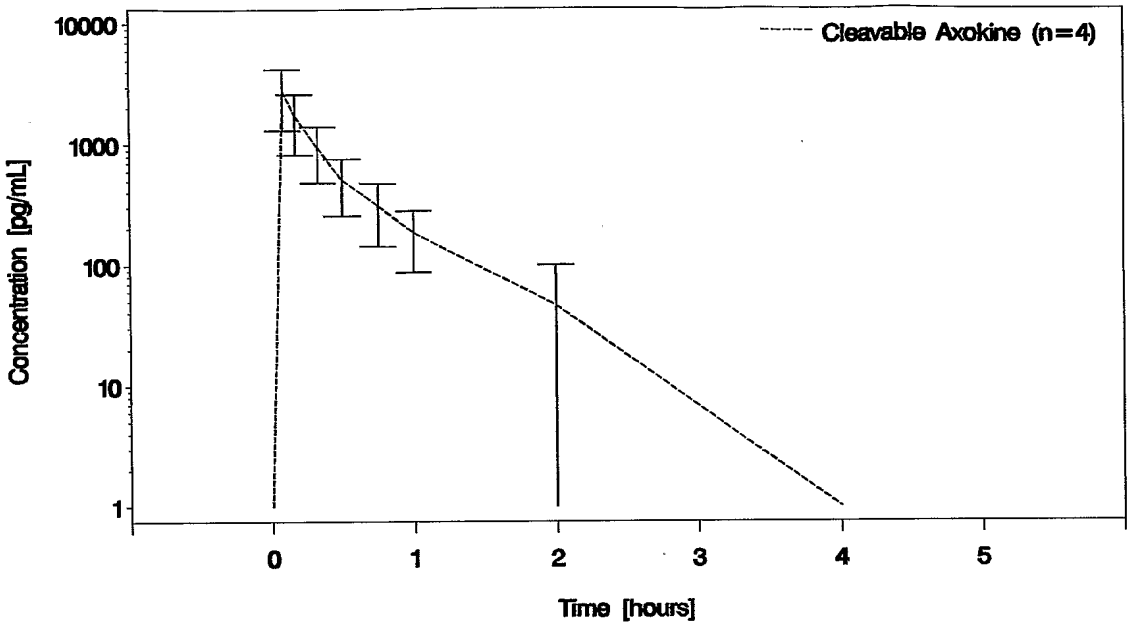


Figure 2: Mean Axokine concentrations +/− SD, following i.v. application

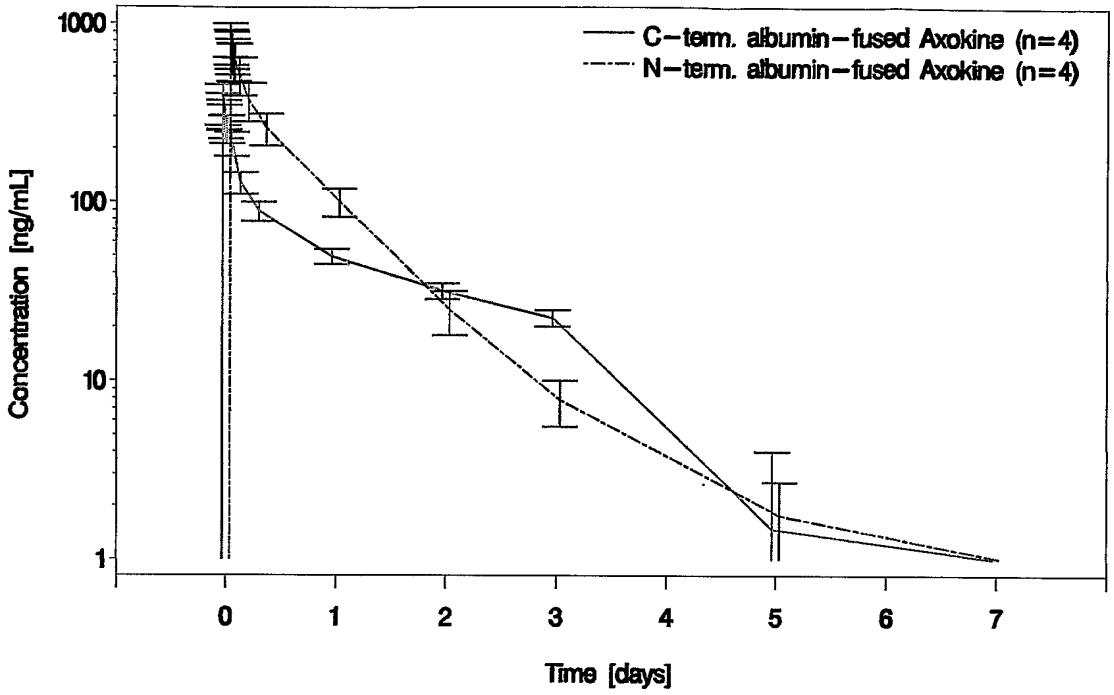


Figure 3: Mean Axokine concentrations +/− SD, following s.c. application

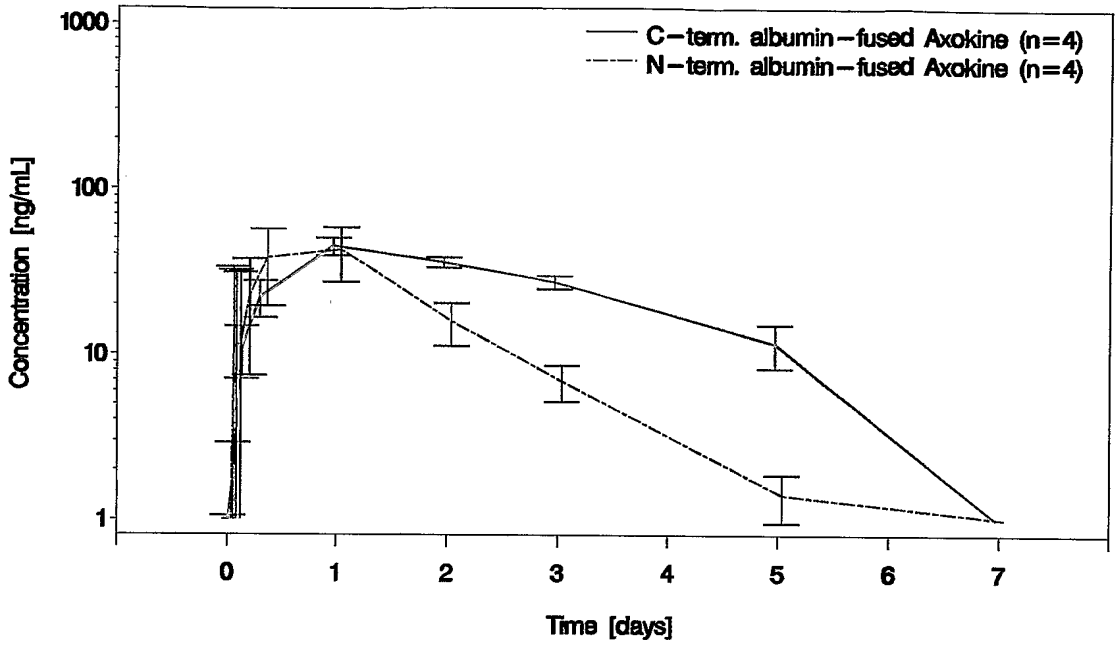


FIGURE 4

Non-fused AXOKINE®in Leptin-Deficient Mice up to Day 102

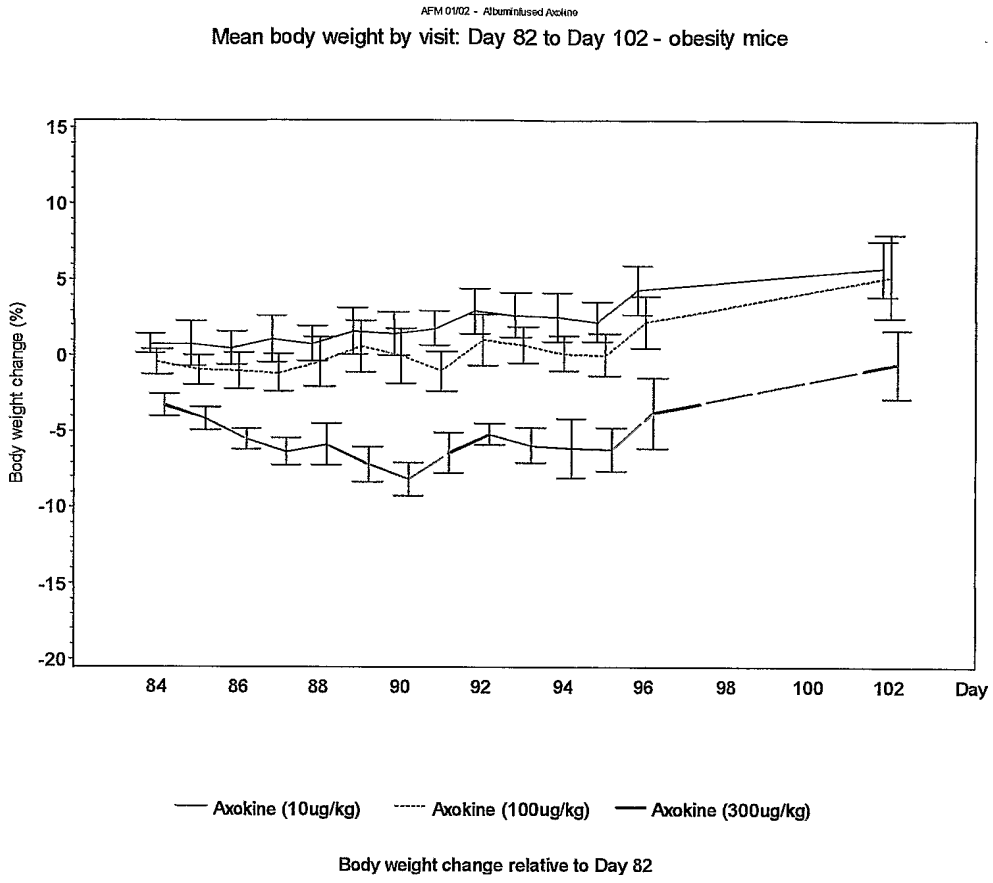


FIGURE 5

Albumin-Fused AXOKINE®in Leptin-Deficient Mice up to Day 102

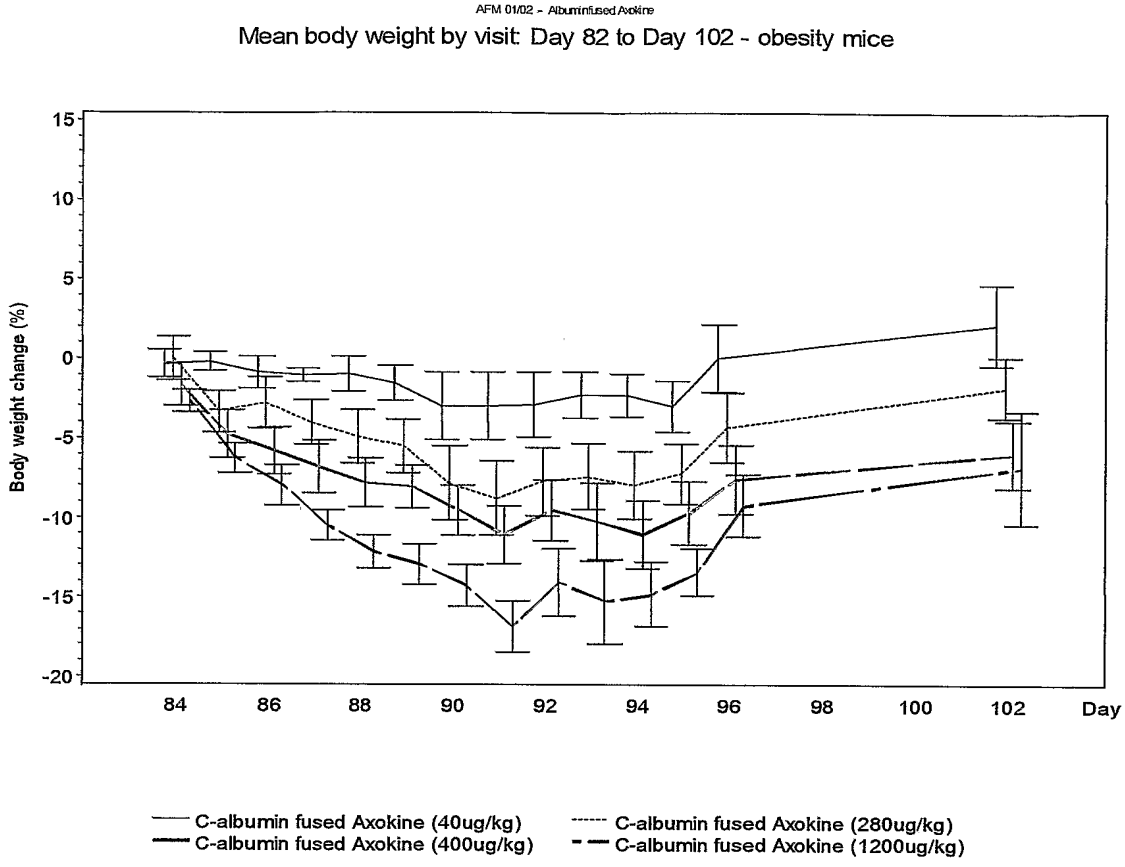


FIGURE 6

Non-fused AXOKINE®in Wild-Type Mice up to Day 102

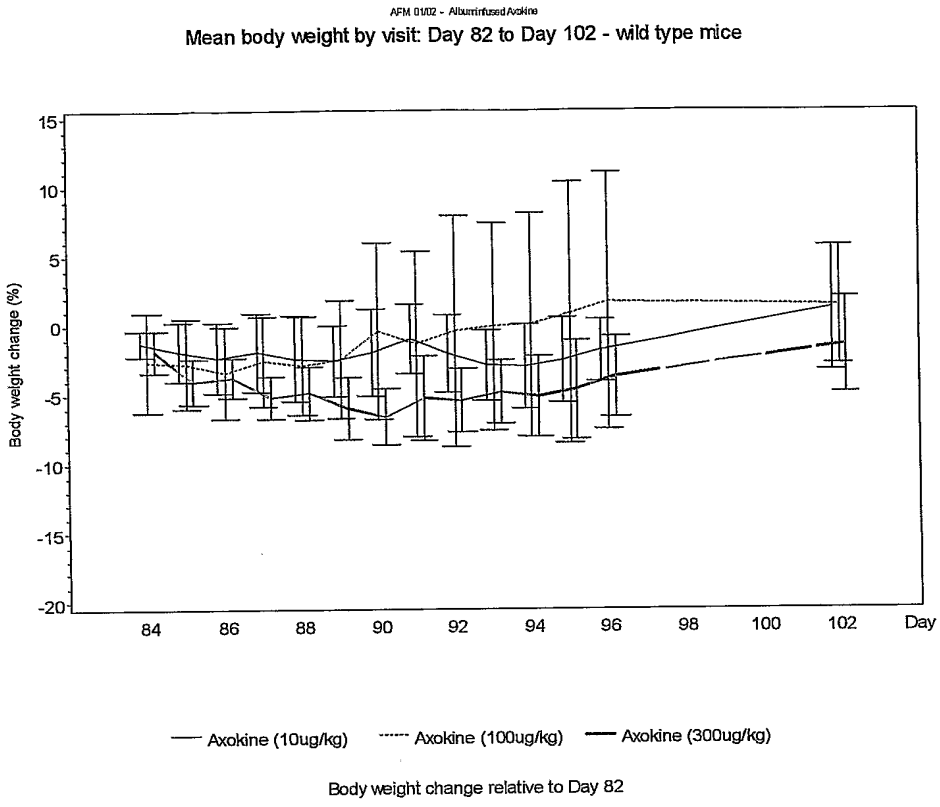


FIGURE 7

Albumin-Fused AXOKINE®in Wild-Type Mice

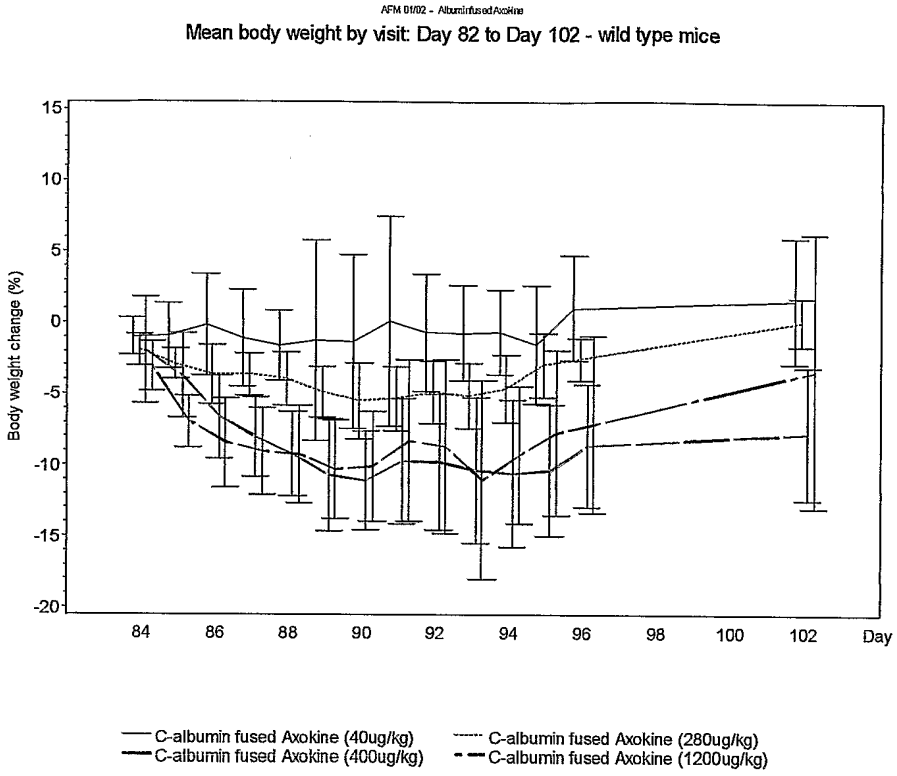


FIGURE 8

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVADES
AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRL
VRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAAD
KAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAE
VSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSH
CIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLL
LRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQ
NALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLC
VLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEK
ERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVA
ASQAALGLGGSGGSGGSGGSGGAFTEHSPLTPHRRDLASRSIWLARKIRSDLTALTE
SYVKHQGLNKNINLDSADGMPVASTDRWSELTEAERLQENLQAYRTFHVLLARLLED
QQVHFTPTEGDFHQAIHTLLLQVAAFAYQIEELMILLEYKIPRNEADGMPINVGDGGLF
EKKLWGLKVLQELSQWTVRSIHDLRFISSHQTG

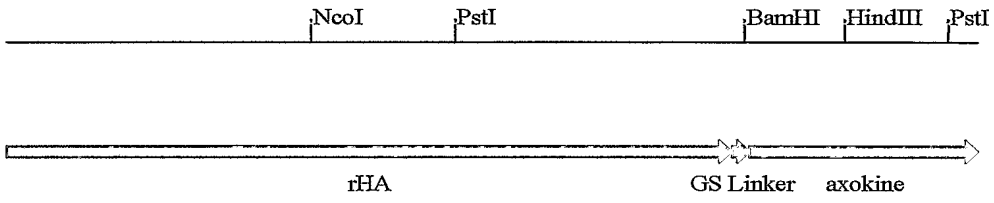
FIGURE 9

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVADES
AENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRL
VRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAAD
KAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAE
VSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSH
CIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLL
LRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQ
NALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLC
VLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEK
ERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLVA
ASQAALGLDYKDHDGDYKDHDIDYKDDDDKAFTEHSPLTPHRRDLASRSIWLARKIRS
DLTALTESYVKHQGLNKNINLDSADGMPVASTDRWSELTEAERLQENLQAYRTFHVL
LARLLEDQQVHFTPTEGDFHQAIHTLLLQVAAFAYQIEELMILLEYKIPRNEADGMPINV
GDGGLFEKKLWGLKVLQELSQWTVRSIHDLRFISSHQTG

FIGURE 10

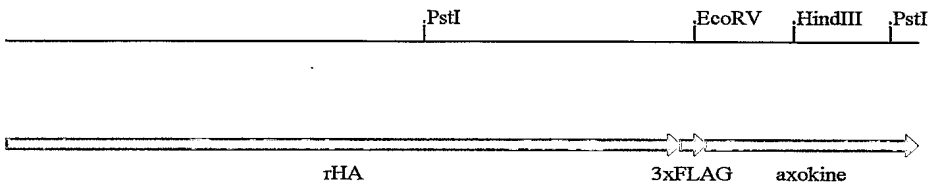
AFTEHSPLTPHRRDLASRSIWLARKIRSDLTALTESYVKHQGLNKNINLDSADGMPVA
STDRWSELTEAERLQENLQAYRTFHVLLARLLEDQQVHFTPTEGDFHQAIHTLLLQVA
AFAYQIEELMILLEYKIPRNEADGMPINVGDGGLFEKKLWGLKVLQELSQWTVRSIHDL
RFISSHQTGGGSGGSGGSGGSGGDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQ
CPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADC
CAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHP
YFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQK
FGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAK
YICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAE
AKDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKP
LVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKC
CKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVD
ETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAA
FVEKCCKADDKETCFAEEGKKLVAASQAALGL

FIGURE 11



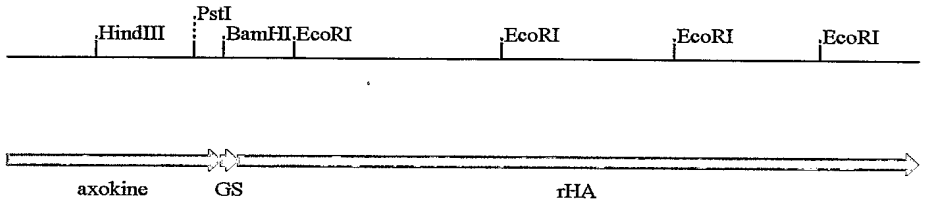
rHA-GS-axokine

FIGURE 12



rHA-3xFLAG-axokine

FIGURE 13



axokine-GS-rHA

FIGURE 14

Figure 14: Weight loss of leptin-deficient (ob/ob) mice during treatment with Axokine/C-terminal albumin-fused Axokine on Days 1, 4, 7

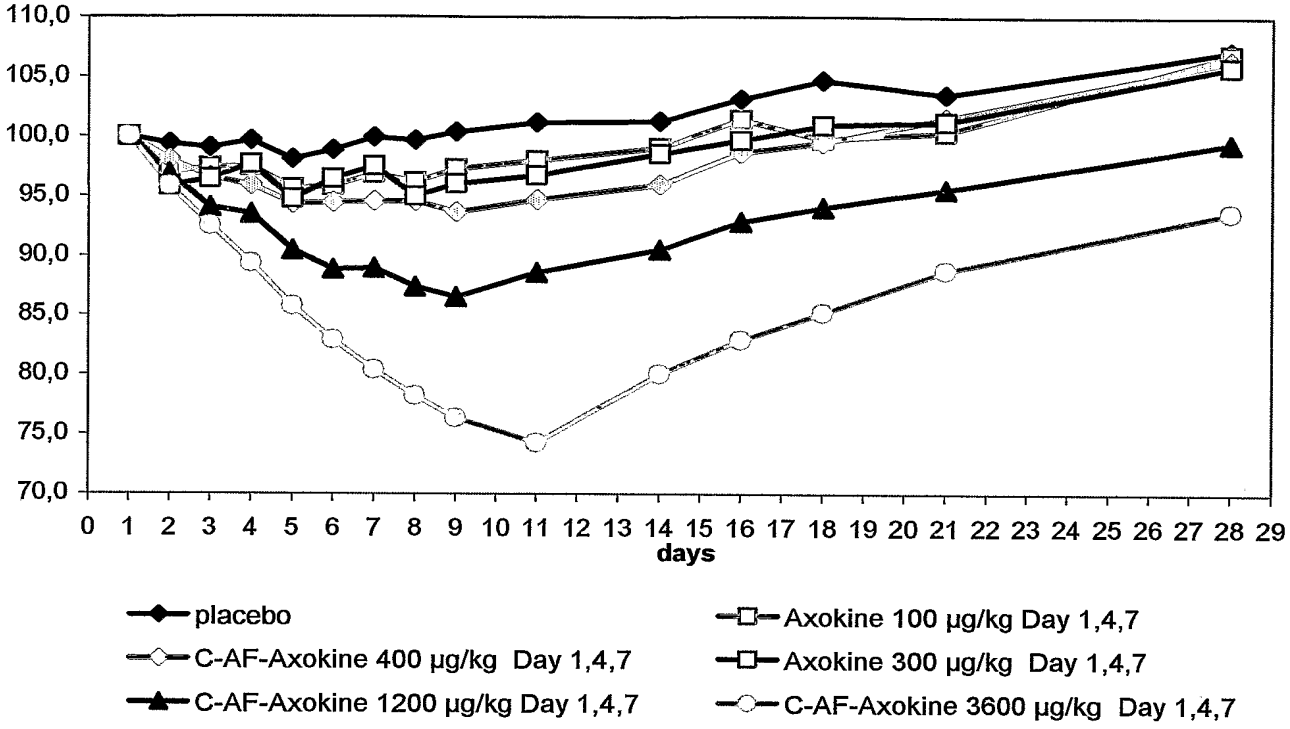
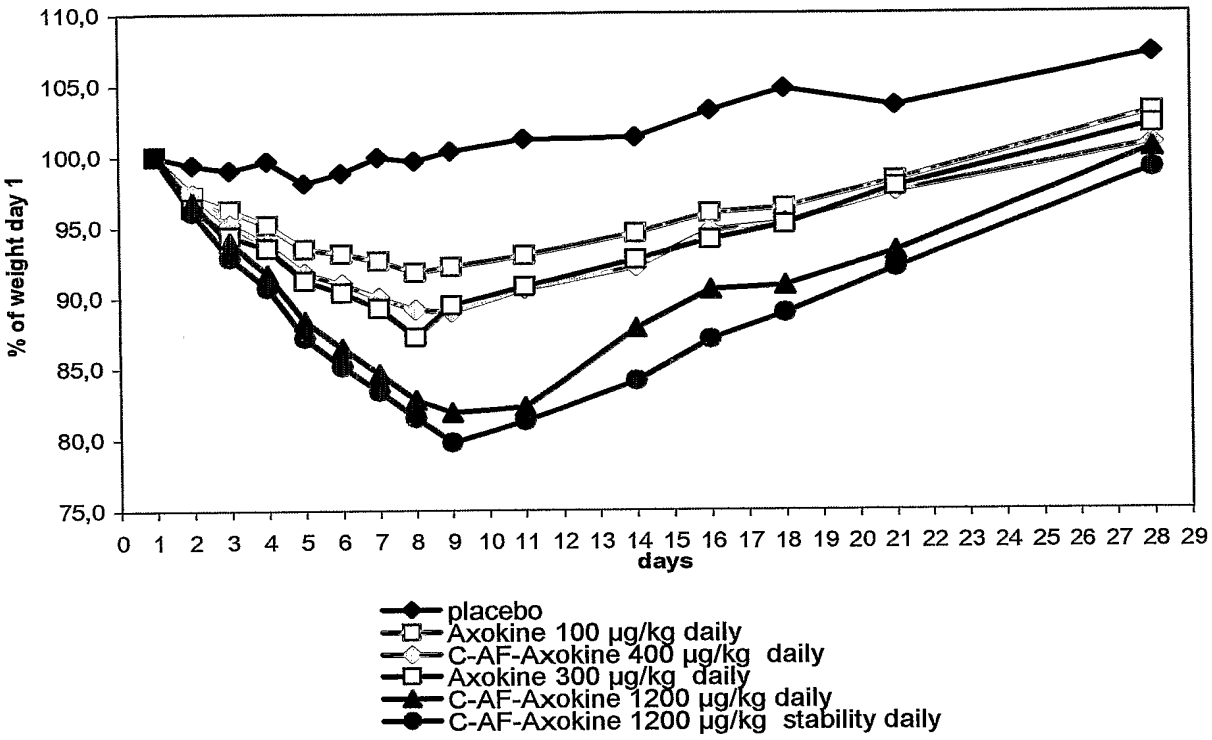


FIGURE 15

Figure 15: Weight loss of leptin-deficient (ob/ob) mice during daily s.c. treatment with Axokine/C-terminal albumin-fused Axokine



Glu Gly Lys Lys Leu Val Ala Ala Ser Gln Ala Ala Leu Gly Leu
770 775 780

2003-M011.ST25.txt
SEQUENCE LISTING

<110> Aventis Behring GmbH

<120> Albumin-Fused Ciliary Neurotropic Factor

<130> 2003M001A058

<140> US 60/401,833
<141> 2003-07-30

<150> US 60/401,833
<151> 2002-08-07

<160> 3

<170> PatentIn version 3.1

<210> 1
<211> 783
<212> PRT
<213> Artificial

<220>
<221> CHAIN
<222> (1)..(783)
<223>

<400> 1

Asp Ala His Lys Ser Glu Val Ala His Arg Phe Lys Asp Leu Gly Glu
1 5 10 15

Glu Asn Phe Lys Ala Leu Val Leu Ile Ala Phe Ala Gln Tyr Leu Gln
20 25 30

Gln Cys Pro Phe Glu Asp His Val Lys Leu Val Asn Glu Val Thr Glu
35 40 45

```
Phe Ala Lys Thr Cys Val Ala Asp Glu Ser Ala Glu Asn Cys Asp Lys
    50                  55                  60

Ser Leu His Thr Leu Phe Gly Asp Lys Leu Cys Thr Val Ala Thr Leu
65                  70                  75                  80

Arg Glu Thr Tyr Gly Glu Met Ala Asp Cys Cys Ala Lys Gln Glu Pro
                85                  90                  95

Glu Arg Asn Glu Cys Phe Leu Gln His Lys Asp Asp Asn Pro Asn Leu
            100                 105                 110

Pro Arg Leu Val Arg Pro Glu Val Asp Val Met Cys Thr Ala Phe His
        115                 120                 125

Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr Glu Ile Ala Arg
    130                 135                 140

Arg His Pro Tyr Phe Tyr Ala Pro Glu Leu Leu Phe Phe Ala Lys Arg
145                 150                 155                 160

Tyr Lys Ala Ala Phe Thr Glu Cys Cys Gln Ala Ala Asp Lys Ala Ala
                165                 170                 175

Cys Leu Leu Pro Lys Leu Asp Glu Leu Arg Asp Glu Gly Lys Ala Ser
            180                 185                 190

Ser Ala Lys Gln Arg Leu Lys Cys Ala Ser Leu Gln Lys Phe Gly Glu
        195                 200                 205

Arg Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser Gln Arg Phe Pro
    210                 215                 220

Lys Ala Glu Phe Ala Glu Val Ser Lys Leu Val Thr Asp Leu Thr Lys
225                 230                 235                 240

Val His Thr Glu Cys Cys His Gly Asp Leu Leu Glu Cys Ala Asp Asp
                245                 250                 255

Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser
            260                 265                 270

Ser Lys Leu Lys Glu Cys Cys Glu Lys Pro Leu Leu Glu Lys Ser His
        275                 280                 285

Cys Ile Ala Glu Val Glu Asn Asp Glu Met Pro Ala Asp Leu Pro Ser
    290                 295                 300

Leu Ala Ala Asp Phe Val Glu Ser Lys Asp Val Cys Lys Asn Tyr Ala
305                 310                 315                 320
```

```
Glu Ala Lys Asp Val Phe Leu Gly Met Phe Leu Tyr Glu Tyr Ala Arg
                325                 330                 335

Arg His Pro Asp Tyr Ser Val Val Leu Leu Leu Arg Leu Ala Lys Thr
            340                 345                 350

Tyr Glu Thr Thr Leu Glu Lys Cys Cys Ala Ala Ala Asp Pro His Glu
        355                 360                 365

Cys Tyr Ala Lys Val Phe Asp Glu Phe Lys Pro Leu Val Glu Glu Pro
    370                 375                 380

Gln Asn Leu Ile Lys Gln Asn Cys Glu Leu Phe Glu Gln Leu Gly Glu
385                 390                 395                 400

Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg Tyr Thr Lys Lys Val Pro
                405                 410                 415

Gln Val Ser Thr Pro Thr Leu Val Glu Val Ser Arg Asn Leu Gly Lys
            420                 425                 430

Val Gly Ser Lys Cys Cys Lys His Pro Glu Ala Lys Arg Met Pro Cys
        435                 440                 445

Ala Glu Asp Tyr Leu Ser Val Val Leu Asn Gln Leu Cys Val Leu His
    450                 455                 460

Glu Lys Thr Pro Val Ser Asp Arg Val Thr Lys Cys Cys Thr Glu Ser
465                 470                 475                 480

Leu Val Asn Arg Arg Pro Cys Phe Ser Ala Leu Glu Val Asp Glu Thr
                485                 490                 495

Tyr Val Pro Lys Glu Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp
            500                 505                 510

Ile Cys Thr Leu Ser Glu Lys Glu Arg Gln Ile Lys Lys Gln Thr Ala
        515                 520                 525

Leu Val Glu Leu Val Lys His Lys Pro Lys Ala Thr Lys Glu Gln Leu
    530                 535                 540

Lys Ala Val Met Asp Asp Phe Ala Ala Phe Val Glu Lys Cys Cys Lys
545                 550                 555                 560

Ala Asp Asp Lys Glu Thr Cys Phe Ala Glu Glu Gly Lys Lys Leu Val
                565                 570                 575

Ala Ala Ser Gln Ala Ala Leu Gly Leu Gly Gly Ser Gly Gly Ser Gly
            580                 585                 590
```

Gly Ser Gly Gly Ser Gly Gly Ala Phe Thr Glu His Ser Pro Leu Thr
595 600 605

Pro His Arg Arg Asp Leu Ala Ser Arg Ser Ile Trp Leu Ala Arg Lys
610 615 620

Ile Arg Ser Asp Leu Thr Ala Leu Thr Glu Ser Tyr Val Lys His Gln
625 630 635 640

Gly Leu Asn Lys Asn Ile Asn Leu Asp Ser Ala Asp Gly Met Pro Val
645 650 655

Ala Ser Thr Asp Arg Trp Ser Glu Leu Thr Glu Ala Glu Arg Leu Gln
660 665 670

Glu Asn Leu Gln Ala Tyr Arg Thr Phe His Val Leu Leu Ala Arg Leu
675 680 685

Leu Glu Asp Gln Gln Val His Phe Thr Pro Thr Glu Gly Asp Phe His
690 695 700

Gln Ala Ile His Thr Leu Leu Leu Gln Val Ala Ala Phe Ala Tyr Gln
705 710 715 720

Ile Glu Glu Leu Met Ile Leu Leu Glu Tyr Lys Ile Pro Arg Asn Glu
725 730 735

Ala Asp Gly Met Pro Ile Asn Val Gly Asp Gly Gly Leu Phe Glu Lys
740 745 750

Lys Leu Trp Gly Leu Lys Val Leu Gln Glu Leu Ser Gln Trp Thr Val
755 760 765

Arg Ser Ile His Asp Leu Arg Phe Ile Ser Ser His Gln Thr Gly
770 775 780

<210> 2

<211> 791

<212> PRT

<213> artificial

<220>

<221> chain

<222> (1)..(791)

<223>

<400> 2

Asp Ala His Lys Ser Glu Val Ala His Arg Phe Lys Asp Leu Gly Glu
1 5 10 15

Glu Asn Phe Lys Ala Leu Val Leu Ile Ala Phe Ala Gln Tyr Leu Gln
20 25 30

Gln Cys Pro Phe Glu Asp His Val Lys Leu Val Asn Glu Val Thr Glu
35 40 45

Phe Ala Lys Thr Cys Val Ala Asp Glu Ser Ala Glu Asn Cys Asp Lys
50 55 60

Ser Leu His Thr Leu Phe Gly Asp Lys Leu Cys Thr Val Ala Thr Leu
65 70 75 80

Arg Glu Thr Tyr Gly Glu Met Ala Asp Cys Cys Ala Lys Gln Glu Pro
85 90 95

Glu Arg Asn Glu Cys Phe Leu Gln His Lys Asp Asp Asn Pro Asn Leu
100 105 110

Pro Arg Leu Val Arg Pro Glu Val Asp Val Met Cys Thr Ala Phe His
115 120 125

Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr Leu Tyr Glu Ile Ala Arg
130 135 140

Arg His Pro Tyr Phe Tyr Ala Pro Glu Leu Leu Phe Phe Ala Lys Arg
145 150 155 160

Tyr Lys Ala Ala Phe Thr Glu Cys Cys Gln Ala Ala Asp Lys Ala Ala
165 170 175

Cys Leu Leu Pro Lys Leu Asp Glu Leu Arg Asp Glu Gly Lys Ala Ser
180 185 190

Ser Ala Lys Gln Arg Leu Lys Cys Ala Ser Leu Gln Lys Phe Gly Glu
195 200 205

Arg Ala Phe Lys Ala Trp Ala Val Ala Arg Leu Ser Gln Arg Phe Pro
210 215 220

Lys Ala Glu Phe Ala Glu Val Ser Lys Leu Val Thr Asp Leu Thr Lys
225 230 235 240

Val His Thr Glu Cys Cys His Gly Asp Leu Leu Glu Cys Ala Asp Asp
245 250 255

```
Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser
            260                 265                 270

Ser Lys Leu Lys Glu Cys Cys Glu Lys Pro Leu Leu Glu Lys Ser His
        275                 280                 285

Cys Ile Ala Glu Val Glu Asn Asp Glu Met Pro Ala Asp Leu Pro Ser
    290                 295                 300

Leu Ala Ala Asp Phe Val Glu Ser Lys Asp Val Cys Lys Asn Tyr Ala
305                 310                 315                 320

Glu Ala Lys Asp Val Phe Leu Gly Met Phe Leu Tyr Glu Tyr Ala Arg
                325                 330                 335

Arg His Pro Asp Tyr Ser Val Val Leu Leu Leu Arg Leu Ala Lys Thr
            340                 345                 350

Tyr Glu Thr Thr Leu Glu Lys Cys Cys Ala Ala Ala Asp Pro His Glu
        355                 360                 365

Cys Tyr Ala Lys Val Phe Asp Glu Phe Lys Pro Leu Val Glu Glu Pro
    370                 375                 380

Gln Asn Leu Ile Lys Gln Asn Cys Glu Leu Phe Glu Gln Leu Gly Glu
385                 390                 395                 400

Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg Tyr Thr Lys Lys Val Pro
                405                 410                 415

Gln Val Ser Thr Pro Thr Leu Val Glu Val Ser Arg Asn Leu Gly Lys
            420                 425                 430

Val Gly Ser Lys Cys Cys Lys His Pro Glu Ala Lys Arg Met Pro Cys
        435                 440                 445

Ala Glu Asp Tyr Leu Ser Val Val Leu Asn Gln Leu Cys Val Leu His
    450                 455                 460

Glu Lys Thr Pro Val Ser Asp Arg Val Thr Lys Cys Cys Thr Glu Ser
465                 470                 475                 480

Leu Val Asn Arg Arg Pro Cys Phe Ser Ala Leu Glu Val Asp Glu Thr
                485                 490                 495

Tyr Val Pro Lys Glu Phe Asn Ala Glu Thr Phe Thr Phe His Ala Asp
            500                 505                 510

Ile Cys Thr Leu Ser Glu Lys Glu Arg Gln Ile Lys Lys Gln Thr Ala
        515                 520                 525
```

```
Leu Val Glu Leu Val Lys His Lys Pro Lys Ala Thr Lys Glu Gln Leu
    530                 535                 540

Lys Ala Val Met Asp Asp Phe Ala Ala Phe Val Glu Lys Cys Cys Lys
545                 550                 555                 560

Ala Asp Asp Lys Glu Thr Cys Phe Ala Glu Glu Gly Lys Lys Leu Val
                565                 570                 575

Ala Ala Ser Gln Ala Ala Leu Gly Leu Asp Tyr Lys Asp His Asp Gly
            580                 585                 590

Asp Tyr Lys Asp His Asp Ile Asp Tyr Lys Asp Asp Asp Asp Lys Ala
        595                 600                 605

Phe Thr Glu His Ser Pro Leu Thr Pro His Arg Arg Asp Leu Ala Ser
    610                 615                 620

Arg Ser Ile Trp Leu Ala Arg Lys Ile Arg Ser Asp Leu Thr Ala Leu
625                 630                 635                 640

Thr Glu Ser Tyr Val Lys His Gln Gly Leu Asn Lys Asn Ile Asn Leu
                645                 650                 655

Asp Ser Ala Asp Gly Met Pro Val Ala Ser Thr Asp Arg Trp Ser Glu
            660                 665                 670

Leu Thr Glu Ala Glu Arg Leu Gln Glu Asn Leu Gln Ala Tyr Arg Thr
        675                 680                 685

Phe His Val Leu Leu Ala Arg Leu Leu Glu Asp Gln Gln Val His Phe
    690                 695                 700

Thr Pro Thr Glu Gly Asp Phe His Gln Ala Ile His Thr Leu Leu Leu
705                 710                 715                 720

Gln Val Ala Ala Phe Ala Tyr Gln Ile Glu Glu Leu Met Ile Leu Leu
                725                 730                 735

Glu Tyr Lys Ile Pro Arg Asn Glu Ala Asp Gly Met Pro Ile Asn Val
            740                 745                 750

Gly Asp Gly Gly Leu Phe Glu Lys Lys Leu Trp Gly Leu Lys Val Leu
        755                 760                 765

Gln Glu Leu Ser Gln Trp Thr Val Arg Ser Ile His Asp Leu Arg Phe
    770                 775                 780

Ile Ser Ser His Gln Thr Gly
785                 790
```

<210> 3

<211> 783

<212> PRT

<213> artificial

<400> 3

Ala Phe Thr Glu His Ser Pro Leu Thr Pro His Arg Arg Asp Leu Ala
1 5 10 15

Ser Arg Ser Ile Trp Leu Ala Arg Lys Ile Arg Ser Asp Leu Thr Ala
20 25 30

Leu Thr Glu Ser Tyr Val Lys His Gln Gly Leu Asn Lys Asn Ile Asn
35 40 45

Leu Asp Ser Ala Asp Gly Met Pro Val Ala Ser Thr Asp Arg Trp Ser
50 55 60

Glu Leu Thr Glu Ala Glu Arg Leu Gln Glu Asn Leu Gln Ala Tyr Arg
65 70 75 80

Thr Phe His Val Leu Leu Ala Arg Leu Leu Glu Asp Gln Gln Val His
85 90 95

Phe Thr Pro Thr Glu Gly Asp Phe His Gln Ala Ile His Thr Leu Leu
100 105 110

Leu Gln Val Ala Ala Phe Ala Tyr Gln Ile Glu Glu Leu Met Ile Leu
115 120 125

Leu Glu Tyr Lys Ile Pro Arg Asn Glu Ala Asp Gly Met Pro Ile Asn
130 135 140

Val Gly Asp Gly Gly Leu Phe Glu Lys Lys Leu Trp Gly Leu Lys Val
145 150 155 160

Leu Gln Glu Leu Ser Gln Trp Thr Val Arg Ser Ile His Asp Leu Arg
165 170 175

Phe Ile Ser Ser His Gln Thr Gly Gly Gly Ser Gly Gly Ser Gly Gly
180 185 190

Ser Gly Gly Ser Gly Gly Asp Ala His Lys Ser Glu Val Ala His Arg
195 200 205

Phe Lys Asp Leu Gly Glu Glu Asn Phe Lys Ala Leu Val Leu Ile Ala
210 215 220

Phe Ala Gln Tyr Leu Gln Gln Cys Pro Phe Glu Asp His Val Lys Leu
225 230 235 240

Val Asn Glu Val Thr Glu Phe Ala Lys Thr Cys Val Ala Asp Glu Ser
245 250 255

Ala Glu Asn Cys Asp Lys Ser Leu His Thr Leu Phe Gly Asp Lys Leu
260 265 270

Cys Thr Val Ala Thr Leu Arg Glu Thr Tyr Gly Glu Met Ala Asp Cys
275 280 285

Cys Ala Lys Gln Glu Pro Glu Arg Asn Glu Cys Phe Leu Gln His Lys
290 295 300

Asp Asp Asn Pro Asn Leu Pro Arg Leu Val Arg Pro Glu Val Asp Val
305 310 315 320

Met Cys Thr Ala Phe His Asp Asn Glu Glu Thr Phe Leu Lys Lys Tyr
325 330 335

Leu Tyr Glu Ile Ala Arg Arg His Pro Tyr Phe Tyr Ala Pro Glu Leu
340 345 350

Leu Phe Phe Ala Lys Arg Tyr Lys Ala Ala Phe Thr Glu Cys Cys Gln
355 360 365

Ala Ala Asp Lys Ala Ala Cys Leu Leu Pro Lys Leu Asp Glu Leu Arg
370 375 380

Asp Glu Gly Lys Ala Ser Ser Ala Lys Gln Arg Leu Lys Cys Ala Ser
385 390 395 400

Leu Gln Lys Phe Gly Glu Arg Ala Phe Lys Ala Trp Ala Val Ala Arg
405 410 415

Leu Ser Gln Arg Phe Pro Lys Ala Glu Phe Ala Glu Val Ser Lys Leu
420 425 430

Val Thr Asp Leu Thr Lys Val His Thr Glu Cys Cys His Gly Asp Leu
435 440 445

Leu Glu Cys Ala Asp Asp Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu
450 455 460

Asn Gln Asp Ser Ile Ser Ser Lys Leu Lys Glu Cys Cys Glu Lys Pro
465 470 475 480

Leu Leu Glu Lys Ser His Cys Ile Ala Glu Val Glu Asn Asp Glu Met
485 490 495

```
Pro Ala Asp Leu Pro Ser Leu Ala Ala Asp Phe Val Glu Ser Lys Asp
            500                 505                 510

Val Cys Lys Asn Tyr Ala Glu Ala Lys Asp Val Phe Leu Gly Met Phe
        515                 520                 525

Leu Tyr Glu Tyr Ala Arg Arg His Pro Asp Tyr Ser Val Val Leu Leu
    530                 535                 540

Leu Arg Leu Ala Lys Thr Tyr Glu Thr Thr Leu Glu Lys Cys Cys Ala
545                 550                 555                 560

Ala Ala Asp Pro His Glu Cys Tyr Ala Lys Val Phe Asp Glu Phe Lys
                565                 570                 575

Pro Leu Val Glu Glu Pro Gln Asn Leu Ile Lys Gln Asn Cys Glu Leu
            580                 585                 590

Phe Glu Gln Leu Gly Glu Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg
        595                 600                 605

Tyr Thr Lys Lys Val Pro Gln Val Ser Thr Pro Thr Leu Val Glu Val
    610                 615                 620

Ser Arg Asn Leu Gly Lys Val Gly Ser Lys Cys Cys Lys His Pro Glu
625                 630                 635                 640

Ala Lys Arg Met Pro Cys Ala Glu Asp Tyr Leu Ser Val Val Leu Asn
                645                 650                 655

Gln Leu Cys Val Leu His Glu Lys Thr Pro Val Ser Asp Arg Val Thr
            660                 665                 670

Lys Cys Cys Thr Glu Ser Leu Val Asn Arg Arg Pro Cys Phe Ser Ala
        675                 680                 685

Leu Glu Val Asp Glu Thr Tyr Val Pro Lys Glu Phe Asn Ala Glu Thr
    690                 695                 700

Phe Thr Phe His Ala Asp Ile Cys Thr Leu Ser Glu Lys Glu Arg Gln
705                 710                 715                 720

Ile Lys Lys Gln Thr Ala Leu Val Glu Leu Val Lys His Lys Pro Lys
                725                 730                 735

Ala Thr Lys Glu Gln Leu Lys Ala Val Met Asp Asp Phe Ala Ala Phe
            740                 745                 750

Val Glu Lys Cys Cys Lys Ala Asp Asp Lys Glu Thr Cys Phe Ala Glu
        755                 760                 765
```